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DR. AND MRS. P. P. LEVINE

A LITTLE over four years ago, *Avian Diseases* made its appearance as a new journal devoted to avian pathology. The editor then and for the first four volumes was Dr. P. P. Levine, New York State Veterinary College, Ithaca, New York. In this undertaking he was ably assisted by many of his co-workers, and was especially encouraged and assisted by his wife. An excerpt from Dr. Levine's report in August, 1960, to the American Association of Avian Pathologists succinctly states the founding editor's vision, hopes, and plans.

"The editor's report consists of the four issues of *Avian Diseases* published every year. If credit be given for the quality of the papers printed, then this credit goes to the authors who had the courage to submit their work to a new and untried journal. If the quality of the manuscripts merits criticism, then the editor will take that blame with a sigh of relief for not having accepted the really poor manuscripts and for taking the trouble to revise the shaky ones. The quality rather than the amount of material printed, measures the worth of a journal. If my successors follow this precept, I'll be more than satisfied. . . .

"My original idea of many years ago was to establish a journal of good quality dealing with our specialty and financially self-sufficient. To a considerable extent these aims have been accomplished. . . ."

The direction given to *Avian Diseases* by Dr. and Mrs. Levine and the original group of associate editors provides guidance and inspiration for their successors.

In dedicating this issue to Dr. and Mrs. Levine and designating P. P. Levine as Honorary Lifetime Editor, the American Association of Avian Pathologists pays tribute to the devotion and vision that made this journal possible.

THE EFFICACY OF CERTAIN COCCIDIOSTATS AGAINST MIXED AVIAN COCCIDIAL INFECTIONS

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Received 1 Nov. 1960

Numerous anticoccidial compounds have been introduced in recent years. As they became available, each has received thorough testing for effectiveness under field conditions. However, information on the comparative efficacy of these anticoccidial agents is incomplete. The contribution made here is designed to fill the deficiency partially. Comparisons are drawn between five anticoccidial compounds, two of them just introduced (1960) on an experimental basis. The other three are well known and commercially available. Additional data are supplied on the effect of graded doses of two coccidiostats on growth and feed efficiency.

PROCEDURE

Five experiments were conducted under various conditions. The first two experiments involved five drugs: Amprol, 1- (4-amino-2-*N*-propyl-5-pyrimidinylmethyl) - 2 - picolinium chloride hydrochloride; Nihydrazone, 5-nitro-2-furaldehyde acetylhydrazone; Nicarbazin, equimolar complex of 4,4-dinitrocarbanilide and 2-hydroxy-4,6-dimethyl-pyrimidine; Unistat, 3,5-dinitrobenzamide and acetyl-(para-nitrophenyl) sulfanilamide; Zoalene, 3,5-dinitro-*o*-toluamide, also called 2-methyl-3,5-dinitrobenzamide. Only Amprol and Zoalene were included in Experiments 3, 4 and 5. Unless otherwise indicated, Amprol, Nicarbazin, and Zoalene were present at 0.0125%, Nihydrazone at 0.011%, and Unistat at .10%. The presence of the various drugs, at the proper levels, was ascertained by analysis in all cases except for Amprol in Experiment 2.

The birds used were fed practical rations containing the individual drugs from one day old. A mixed culture of oocysts of predetermined constitution was administered directly into the crop of each bird with a pipette. The birds were weighed as a group at the time of infection, and each group was reweighed when birds were removed for examination.

Broiler-type chicks used in Expts. 1 and 2 were grown in batteries with raised wire floors. Oocysts were administered at age 3 weeks. The efficacy of each drug (anticoccidial index) was expressed by the equation: percent survival + percent gain of initial weight – lesion score – oocyst score.

Lesion score was based on macroscopic examination of the duodenum, small intestine, and ceca. The score was determined at 0, 1, 2, 3, or 4, based on severity of gross lesions observed. Oocyst score was determined by microscopic examination of scrapings from the same three intestinal areas, and the score assigned based on the relative number of oocysts present.

Percent survival was calculated at nine days post-challenge. The lesion and oocyst scores were compiled from examinations made of sample birds from each lot on the fifth, sixth, and ninth days post-challenge. Percent gain of initial weight in Expt. 1 involves the total gain of all chicks including those removed for examination on the fifth and sixth days, whereas percent gain of initial weight in Expt. 2 was calculated on weight at nine days post-challenge.

Studies to determine the effect of graded levels of Amprol and Zoalene on growth and feed efficiency were made in Expts. 3 and 4. The drugs were present at 0.0125%, 0.0150%, and 0.0250%. Broiler-type chicks were used and weight determined at five weeks of age.

The purpose of Expt. 5 was to determine the immunity that had developed when replacement birds were fed Amprol and Zoalene at two levels until 12 weeks old. Chicks from a laying strain (Hylines) were used in this experiment, and each treatment consisted of two replicates of 150 birds per replicate. Sample birds were removed from the experimental diets and placed on the basal diet at the time of infection. Prior to challenge the birds were raised on litter, but at the

time of infection were placed on wire floors. Fresh shavings was the litter used, but the pens had contained birds during previous experiments, thus affording opportunity for a natural exposure.

Ten birds were removed from each replicate when 8 weeks old and challenged with a mixed culture of oocysts. Seven additional birds were taken from each replicate when 12 weeks old, and the challenge was repeated. Litter counts were made when birds were removed at both 8 and 12 weeks.

To determine the effect of Amprol fed to replacement birds for an extended period, pullets from this experiment were carried through on two levels of Amprol (0.008% and 0.004%) until 23 weeks old. The birds then received a complete laying ration, and at 30 weeks of age five birds from the lot formerly on 0.008% Amprol and five birds from the basal group were examined histopathologically.

Table 1. Relative efficacy of anticoccidial compounds against challenge[a] with mixed culture of oocysts.

Drug	Level (%)	Survival[b] (%)	Gain (%)	Lesion score	Oocyst score	Anticoccidial[c] index
Expt. 1[d]						
Basal	—	95	58	5.6	0.2	141.2
Amprol	0.0125	100	75	2.3	2.7	170.0
Nicarbazin	0.0125	100	71	3.3	1.5	166.2
Zoalene	0.0125	100	57	4.5	6.4	146.1
Unistat	0.10	95	36	6.6	6.6	117.8
Nihydrazone	0.011	95	16	5.3	6.6	99.1
Expt. 2[d]						
Basal	—	80	3	5.1	5.5	72.4
Amprol	0.0125	100	65	2.3	1.6	161.1
Nicarbazin	0.0125	100	60	2.0	2.7	155.3
Zoalene	0.0125	100	61	3.4	5.3	152.3
Unistat	0.10	95	33	4.8	5.8	98.4
Nihydrazone	0.011	90	19	4.8	5.8	98.4

[a] Challenge given at three weeks of age.

[b] 20 birds per lot.

[c] The larger the anticoccidial index the more effective the coccidiostat.

[d] Oocysts administered per bird:

	Expt. 1	Expt. 2
E. acervulina	—100,000	75,000
E. tenella	—130,000	150,000
E. maxima	— 96,000	75,000
E. necatrix	—125,000	150,000
E. brunetti	—100,000	75,000

RESULTS

Expt. 1. From Table 1, two of the drugs, Amprol and Nicarbazin, seemed superior to Unistat and Nihydrazone and somewhat more effective than Zoalene. Though the basal group responded in an atypical manner, the order of the coccidiostats, based on efficacy, is the same as that obtained in a preliminary trial and agrees with that found in Expt. 2.

Expt. 2. The results (Table 1) of Expt. 2, were similar to those of Expt. 1. The basal group followed the expected pattern, with 20% mortality and essentially zero gain. Amprol, Nicarbazin, and Zoalene gave very similar results, and appeared much more effective than Unistat or Nihydrazone.

Expt. 3. Table 2 contains the results obtained when twice and four times recommended levels of Zoalene were fed. Growth and feed efficiency were somewhat depressed at 0.025% and very markedly so at 0.05%.

Expt. 4. Table 2 indicates the results obtained when Amprol was fed at twice and four times recommended levels. No depression of either growth or feed efficiency was noted at any level used in this experiment.

Expt. 5. The response of sample birds challenged at eight and twelve weeks did not provide a distinct pattern. Mortality was heavy in all groups. In all of the groups in which coccidiostats had been included there were less than 10,000 oocysts per gram of litter at both eight and twelve weeks; 40,000 and

Table 2. Effect of graded levels of Zoalene and Amprol on growth and feed efficiency of chicks at five weeks of age.

Level (%)	Weight[a] (g)	Efficiency[b]
Expt. 3 (Zoalene)		
0	490	1.94
0.0125	499	1.94
0.0250	449	2.05
0.050	281	2.32
Expt. 4 (Amprol)		
0	581	1.86
0.0125	590	1.80
0.0250	590	1.84
0.050	608	1.81

[a] Three replicates per treatment, 7 males and 7 females per replicate.
[b] Units feed per unit gain.

30,000 oocysts per gram were found in the two basal groups at eight weeks. One basal group at twelve weeks yielded 20,000 oocysts per gram of litter and the second basal group had less than 10,000 oocysts per gram.

The birds that had received Amprol until 23 weeks old gave no evidence of histopathological changes. During two months of production no differences have been noted in percent production, shell quality, or egg size between the birds fed the basal ration and those that had received Amprol.

DISCUSSION

Amprol, Nicarbazin, and Zoalene were obviously superior coccidiostats in these experiments. Unistat and Nihydrazone both proved much less effective, and for this reason were not included in the studies on the replacement birds. Nicarbazin was not studied further, because earlier work had indicated that Nicarbazin, though an effective coccidiostat,[3] may, under certain conditions, adversely affect production,[8] and is quite toxic at increased levels.[5] Cuckler and Malanga,[2] Ott *et al.*,[6] and McLoughlin *et al.*[4] found that Nicarbazin allowed immunity to develop, though Berg *et al.*[1] indicated that immunity was not developed against *E. necatrix* when Nicarbazin was the coccidiostat.

As pointed out by Peterson,[7] the desirable characteristics of a coccidiostat vary with the purpose for which the bird is being raised. It is quite possible that Unistat or Nihydrazone would perform satisfactorily when used for replacement birds. It has been our experience, however, that drugs that fail to withstand a challenge such as used here, also fail to prevent coccidiosis in the field, where exposure is often heavy.

At the time these trials were carried out, Zoalene and Nicarbazin were known to be effective coccidiostats since both were being used commercially in Canada. Thus, a coccidiostat that approximated the response obtained with either of these drugs was assumed to be effective. This procedure was used by Peterson.[7]

Zoalene appears to be tolerated over a rather narrow range, although, for practical purposes, the range is sufficient to give small cause for concern. Amprol gave no evidence

of being toxic, even at four times the level recommended for broiler chicks. It should be noted that the level at which Amprol might depress growth will vary with the thiamine level of the diet. The toxicity of the other coccidiostat effective in these trials, Nicarbazin, has been studied by Newberne *et al.*[5] who indicated that a level of 0.025% depresses growth.

The build-up of oocysts in the litter in Expt. 5 was so slight that even the birds in the basal group did not become immune. Earlier, in previous experiments under similar conditions, a solid immunity developed in the basal group at six weeks of age, whereas birds on coccidiostats were still susceptible to the challenge. No conclusions can be drawn as to the development of immunity in chickens receiving either Zoalene or Amprol at the levels used in this work.

Only Amprol was fed to replacement birds until 23 weeks old. It was deemed unnecessary to feed Zoalene in this manner because the latter has been widely used in Canada since 1958 and it has been amply demonstrated that no adverse effect on egg production has resulted, even when Zoalene has been included in the diet until pullets are well into production.

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The authors thank Mr. O. Remmler and Mr. John Watters for technical assistance. The authors also express appreciation for experimental materials, for analysis of various diets, and for determination of the oocyst count in the litter to Merck and Company, Montreal. Thanks are also due VioBin (Canada) Limited, St. Thomas, Ontario; Dr. Salsbury's Laboratories, Charles City, Iowa; and Dow Chemical Company of Canada, Limited, Sarnia, Ontario, for experimental materials and analyses of various diets.

SUMMARY AND CONCLUSIONS

1. Amprol, Nicarbazin, and Zoalene appear to be effective coccidiostats in two battery experiments using an anticoccidial index of efficacy after challenge with *E. acervulina*, *E. tenella*, *E. maxima*, *E. necatrix*, and *E. brunetti* in standardized doses of oocysts. Under these conditions Nihydrazone and Unistat appear much less effective.

2. Amprol has a wide margin of safety when growth and feed efficiency are used as the criteria. Zoalene appears to be tolerated over a much narrower range.

3. Amprol, when fed from 1 day old to 23 weeks old, at either 0.004% or 0.008%, caused no apparent histopathological changes and had no effect on egg production during the first two months of lay.

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EXPERIMENTAL LISTERIOSIS OF TURKEYS

F. M. BOLIN and D. F. EVELETH

Received 12 September 1960

IN HIS REVIEW of listeriosis of fowls Gray[1] lists only two references on listeriosis of turkeys. Belding and Mayer[2] reported two naturally occurring field cases diagnosed at Michigan State University. In one case, 18-week-old birds showed torticollis with tremors of the skeletal muscles. The gross pathology consisted of a mild hyperemia of the cerebral hemispheres. *Listeria monocytogenes* was isolated from the brain tissue.

In the second case reported by these authors, no symptoms were observed. The only gross lesions found on necropsy were white necrotic foci on the liver. A greenish discoloration of the lung was considered to be due to post-mortem change. *L. monocytogenes* was isolated from the visceral organs.

Malewitz[3] et al. investigated experimentally induced listeriosis in turkey poults. Poults 3 to 11 days old were used in their study. They were inoculated intraperitoneally with a broth culture of *L. monocytogenes*. Drooping of the head, followed by nervous symptoms, was observed in 16–20 hours. Paralysis followed, and all birds died within 24–80 hours.

At necropsy it was observed that the livers appeared mottled and the epicardium was hemorrhagic, but there were no gross lesions in the brain.

Listeriosis is a serious problem in sheep and cattle in North Dakota and has been diagnosed in swine, chickens, and a skunk. Many turkeys are submitted to this laboratory for diagnosis, but *L. monocytogenes* has never been isolated from any of these birds. The possibility that this disease is present in turkeys and not being diagnosed was considered, although nearly all birds are routinely cultured.

This is a progress report on Project H-16-5 M and is published with the approval of the Director of the North Dakota Agricultural Experiment Station, Fargo, North Dakota.

The results of experimentally induced listeriosis are summarized in this report.

MATERIALS AND METHODS

The turkeys used were Broad Breasted Whites from a commercial hatchery but raised in this laboratory. There were no death losses, nor were there any clinical symptoms of an infectious disease before the start of the experiment. The experiments were started when the poults were first inoculated, at 15 weeks old.

The inoculum was a broth culture of *Listeria monocytogenes* Type 1,[4] originally isolated from a sheeep and later inoculated into and reisolated from a bovine. There were no physical changes in the cultures on artificial media, and the strain was pathogenic when inoculated into the guinea pig eye and had a high pathogenicity when used in 2 successive bovine listeriosis experiments.

A 24-hour broth culture was used in all cases. One ml (which contained 54 billion organisms, established by dilution and plate-culture colony counts) of the broth culture was injected intravenously at each challenge. Twenty poults received 2 inoculations 7 days apart, and 20 other poults received three challenge doses 7 days apart. Six birds were left in contact with the inoculated, but received no direct challenge.

RESULTS

There were 11 deaths during the 60 days following the first challenge. *Listeria monocytogenes* was isolated from 7 of these 11 poults. Death resulted in 3 of 6 twice-injected poults, and 3 of 5 receiving three injections.

The length of time from inoculation until death of the birds from which Listeria was recovered varied from 14 to 52 days.

The four from which Listeria was not isolated on postmortem had lesions of a massive airsacculitis and peritonitis from which staphylococci were recovered. The remaining 29 poults were killed at two months post-inoculation, and in no case was Listeria recovered.

All of the poults that died and from which Listeria was

recovered showed evidence of hepatitis. The gross lesions varied from focal necrosis to green discoloration. Most of the poults had enlarged spleens with a mottled appearance. Emaciation and diarrhea were found in nearly all of the inoculated birds.

DISCUSSION AND SUMMARY

The results indicate that severe challenge with *Listeria monocytogenes* cultures by intravenous inoculation caused death in only 17.5% of the poults inoculated at 15 weeks of age. There was no appreciable difference between the groups. None of the six contact controls developed listeriosis.

The enlarged and mottled spleens found in both the poults from which Listeria was isolated and those which were negative for Listeria is suggestive that the pathologic changes may have been caused by the bacterial infection but that in those cases from which no viable Listeria could be cultured the body defenses had overcome the infection.

The results of experimental challenge of turkey poults indicate that listeriosis of turkeys is not likely to be of epizoologic importance under these conditions.

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CANDIDIASIS IN TURKEYS AND CHICKENS IN THE SACRAMENTO VALLEY OF CALIFORNIA[a]

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Received 27 April 1961

CANDIDIASIS diagnoses have been infrequent in diagnostic laboratory reports from various states.[10] Underwood *et al.*[44] regarded the disease as an insignificant problem of poultry in the U. S. except for sporadic outbreaks. In California, these assessments may be true for most of the state except in the Sacramento Valley.

Annual summary reports covering 1955–60 from the California Department of Agriculture's 6 diagnostic laboratories[7] listed the following case incidence of this entity (unspecified "mycoses" were not included): San Gabriel, 22; Lancaster, 14; Fresno, 0; Turlock, 3 (all in 1960); Petaluma, 17; and Sacramento, 206.

The Sacramento Valley also figured in the early history of this disease on this continent. In 1931, shortly after Jungherr,[22] in Connecticut, recognized the disease for apparently the first time in this country. Gierke,[14] at the state department of agriculture diagnostic laboratory at Sacramento, and Hinshaw,[15] at the University of California at Davis, working independently, encountered this disease as a distinct problem in poultry in the Sacramento Valley.

Eberth,[10] in 1858 in Europe, was perhaps the first to report the disease in poultry after Langenbeck[45] reported the first human case, in 1839. In Great Britain, the initial accounts of this disease in poultry were published in 1949[6] and 1950.[3] But Wilson[47] claimed, in 1949, that he had diagnosed the disease in Scotland in 1934. It has been reported as a poultry problem in Ceylon in 1953,[30] in Australia in 1957,[45] and in Thailand in 1956.[26]

The decision at the Sacramento laboratory to restudy individual records of relatively recent consignments in which candidiasis was a significant factor was influenced by reports of incidence paucity and the reported current upsurge of interest in *Candida*.[40] The data below were obtained from a restudy of 167 consignments received in the four years 1957–60.

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[a] Presented at 10th Western Poultry Disease Conference, Feb. 1, 1961, Davis, California.

Incidence in the Valley

The geographical incidence was as follows: In 1957, 11 growers in the adjoining counties of Placer, Nevada, and Sacramento, encountered the disease. In 1958, 19 growers experienced the problem in Sacramento, Placer (which had 12 growers alone), and in the adjoining counties (to the south) of El Dorado, San Joaquin, and Stanislaus. Twenty-five growers in 1959 and 18 in 1960, in virtually every county within the valley, which extends over 200 miles, experienced the mycosis.

Seasonal-incidence highlights were as follows: 43 consignments were received during July, which made it the heaviest-incidence month (26% of the total). Eighty-five percent of the consignments were received during the summer and fall months.

These findings are strikingly similar to the seasonal incidence reported in 1931. Jungherr[22] noted that his outbreaks appeared around the middle of April, peaked during June and July, and lasted through August. Gierke[14] reported that his outbreaks appeared during the last week of June and continued through July. Hinshaw[15] reported that his outbreaks had origin in the fall of that year. Candidiasis was encountered in turkeys in Pennsylvania in the summer of 1959.[13] These seasonal findings, generally similar to those reported for coccidioidomycosis,[28] suggest the probability that candidiasis may be a dust-borne disease, as is coccidioidomycosis.

The age incidence study revealed that 80% of the 101 turkey consignments comprised turkeys within their first 3 months of life, which is similar to findings of other workers. Gierke[14] noted that 2–3-month-old turkeys were primarily affected, and Hinshaw[15] observed acute outbreaks in 2–3-month-old turkeys. Jungherr[23] observed that turkeys under a month of age were hardest hit. Chute[10] considered young birds more susceptible than old birds. Of the 66 chicken consignments, 64% comprised birds 7–10 months old. No cases involved chicks in their first month, as experienced by Jungherr.[22] In Great Britain a decided incidence increase with age was reported in chickens.[21]

Etiology

Candida has been almost universally accepted as the generic term for this yeast-like fungus; however, the term "moniliasis" is still used occasionally[44,45,50] in reference to disease caused by *C. albicans.* Vanbreuseghem[45] maintained that Candida is a true yeast; therefore, the resultant disease is a "levurosis," which category also includes cryptococcosis *(Cryptococcus neoformans),* a cause of meningitis in man. However, the consensus is that it is a "yeast-like fungus" because of the presence of mycelium. But some strains of *Candida* may be completely devoid of mycelium, even in exacting mycelium-producing media, until after a few years of cultivation.[40] Therefore, such strains of *Candida* have been erroneously called true yeast or a *Cryptococcus;* later they were identified as yeast-like

Candida.[40] As a general rule, when growth conditions are optimal, the S (or yeast) phase predominates, and when suboptimal, the R (or mycelial) phase predominates.[39] Acidity favors phase-S growth, and alkalinity favors phase-R growth.[45] When small amounts of glucose are added to a purified polysaccharide medium, both filamentation and chlamydospore formation are suppressed.[29]

The morphologic components of this fungus are: the round or elliptical blastospores, 2–4 μ in size, hyphae, and, only within the *C. albicans* group,[45] chlamydospores, 7–8 μ (or more) in diameter, double-contoured, refractive, acid-fast, and usually arising at ends of filaments. Chlamydospore production criterion is therefore of critical importance because *C. albicans* is generally regarded as the pathogenic *Candida*.[11,40]

A trial was conducted to determine viability of organism *in situ* in sub-zero cold. A portion of a diseased crop, in a covered Petri dish, was stored 25 months at –20°C, and then thawed, and mucosal scrapings were inoculated onto Pagano-Levin medium (E. R. Squibb, New York City), Luxuriant growth of *C. albicans* was obtained within 48 hours of incubation.

There are 172 synonyms for *C. albicans*.[11]

Pathology

The crop was the most common site of infection and was marked with the characteristic "turkish towel" pseudomembranous lesions in the severe stage. When the skin over the crop was reflected, a severely attacked organ had a suggestive cooked appearance. Esophageal, pharyngeal, palatine, glossal, and proventricular involvements were found occasionally. Buccal, pharyngeal, and esophageal lesions were usually described as ulcerative pseudomembranous patches. A severely attacked proventriculus was characterized by marked swelling of the entire organ, and edema, catarrh, necrosis, and ulcerations of the mucosa.

On premise CB (Placer County), where candidiasis in turkeys has been a perennial problem, the outbreaks of January, April, and May of 1960 were marked by a consistent association of focal (pinpoint to pinhead-size grayish lesions) hepatitis with candidiasis of the upper alimentary tract. Unfortunately, the basic diagnostic approach for the hepatopathy was bacteriological, so the fungal regard was slighted. In September of 1960 Gentry[13] reported severe hepatopathy in turkeys, characterized by severe necrotic and granulomatous lesions, with *C. albicans* being isolated in pure cultures from tissues surrounding these lesions, in most of the crop candidiasis cases encountered in several areas of Pennsylvania in the summer of 1959. Jungherr[22] reported that in his 1931 experiences, 6 of 120 field cases had liver involvement.

Diagnostic Procedures and Problems

The manner of crop examination is held as important in diagnosing candidiasis, because of the following considerations: the

crop is the organ most commonly attacked; "mushy" ingesta are usually present except in the anoretic state; lesions may sometimes be confined to the ventral aspect of the organ; there is a tendency for examiners to slit the organ open (without excision) and to scrape out the ingesta casually with a blade as an inspection prelude; pseudomembranes of an acute case are characteristically readily scraped off; lesions may be pinhead in size;[22,43] early stages are unspectacular; and if a negative impression is gained by casual inspection of the crop, check of the rest of the upper alimentary tract may be slighted.

The method adopted for routine examination of the crop and portions of the esophagus is as follows: excise out entire crop and some of upper and lower esophagus, slit open, shake out loose ingesta, and then wash off all adherent ingesta with a gentle water spray. The mucosal surface of the organs is thus prepared for visual inspection, culturing, microscopic examination of lesions, and histological sectioning, if necessary.

For culturing, the Pagano-Levin medium was satisfactory for presumptive differentiation of *Candida*. Differentiation is based upon the varying ability of yeasts and fungi to reduce a tetrazolium compound present to insoluble, colored formizans.[25] The bacterial inhibitor, neomycin, has been efficient provided the second swab (rubbed over same area as the first) is used for inoculation.[42] For determination of chlamydospore production, rice infusion agar has been used. The identification of *C. albicans* on the basis of chlamydospore presence was discussed by Bakerspigel.[1]

In preparing mucosal scrapings for microscopy, clearing was not necessary. The addition of potassium hydroxide solution to scrapings is objectionable because, in itself, it is capable of production of yeast-like artifacts.[45] If tissue invasion by the fungus was questioned, the selections were sectioned and stained with the Gridley fungal stain for histological viewing. Tissue invasion was demonstrated in virtually all areas of the upper alimentary tract.

In numerous cases ultimately diagnosed as "enteritis" there was no significant inflammation of the upper alimentary tract mucosa but the small intestinal mucosa manifested a proliferative necrotic reaction in the severe stage. Microscopy of such mucosal scrapings revealed dense infiltration of blastospore-like bodies basically within the tips of villi, with hyphae not being apparent. However, this fungal suspicion has not yet been confirmed by the culturing and histological means employed. On past occasions, a peculiar necroproliferative typhlitis has been found as the basic pathology, in which the "neck" portion was involved. Microscopy of such tissue scrapings has revealed the dense presence of mycelium-like structures growing out from the mucosal surface like an unkempt head of hair. Examination of fresh tissue and hematoxylin eosin–stained sections have demonstrated superficial tissue invasion; again without conclusive confirmation.

Jungherr,[22] in the east, thought that some hens had vent involvement, though it is unclear whether histological evidence was obtained, but Hinshaw[15] and Gierke,[14] in the west, did not encounter intestinal candidiasis. Blaxland and Markson,[4] in England, could not demonstrate intestinal candidiasis histologically. Underwood[44] did not find definitive lesions on gizzard lining or intestinal mucosa in experimentally infected birds.

Our isolates have produced striking pathology and death in rabbits, as described by some workers.[11,39,45] Vanbreuseghem[45] considered that *C. tropicalis* is the only other *Candida* pathogenic for the rabbit, though of a lesser magnitude than *C. albicans*. Virulence enhancement of *C. albicans* after growth on media containing cysteine has been established.[49] Therefore, for accurate assessment of pathogenicity, fungal cultures intended for animal transmission trials should be propagated in media devoid of this amino acid. The pathogenicity of this fungus for Swiss mice has been demonstrated.[33]

Ecology and Pathogenesis

The "dual personality" nature of *C. albicans*—a paradox of being a commensal on the one hand and a parasite on the other—is intriguing. In this respect, it has been likened to *Staphylococcus* by some workers.

It was mentioned as a commensal in man by some authors,[11,40,45] and in poultry by other authors.[4,21,27,34] However, it was considered as the only species of *Candida* that is definitely pathogenic.[40] True human epidemics have been recorded.[11] Diddens and Lodder (regarded by Skinner *et al.*[39] as "specialists in yeast-like fungi") classified this species as one of 12 pathogenic *Candida* species. Yacowitz *et al.*[50] found this disease in poultry to be highly contagious. Gentry *et al.*[13] encountered this fungus as a pathogen in Pennsylvania turkeys. Blaxland and Fincham[3] reported on the primary-pathogen nature of their poultry isolates.

The relations of avitaminosis-A to candidiasis was shown in a study when only one chicken of 14 on a balanced diet developed mild candidiasis, whereas 62% of chickens on a vitamin-A-deficient diet developed severe candidiasis.[34]

Breed susceptibility differences were shown in a 10-week test period when, under the same conditions, Broad-Breasted Bronze turkeys manifested pendulous crops and 50% mortality, and Beltsville Small White turkeys did not have pendulous crops but mortality was 100%.[46]

The role of the reported endotoxin of *C. albicans* in the pathogenesis of the disease is another intriguing subject. Salvin[32] demonstrated that *C. albicans* had a soluble endotoxin which was lethal for mice. Roth andMurphy[31] demonstrated that a cell-free extract (lysate) of *C. albicans* (obtained by supersonic vibration) was lethal for mice pretreated with chlortetracycline.

In chickens, particularly in those caged, this disease has been noticeably associated with waterer insanitation, as reported by others.[3,10,15,16,26] The pressure of this disease problem has stimulated some improvement in waterer sanitation practices in this region.

The reported influence of antibacterial antibiotics on the pathogenesis of candidiasis has been a stimulant in the recent upsurge of interest in this disease.[40] Chlortetracycline significantly stimulated the growth of *C. albicans* in vitro.[19] Chlortetracycline enhanced the virulence of *C. albicans* in mice,[36,37] and so did oxytetracycline.[37] Penicillin enhanced the pathogenicity of *C. albicans* var. *Stellatoidea* in chick embryos and rabbits.[12] Chlortetracycline, neomycin, and bacitracin stimulated the *in vitro* growth of *C. albicans*.[20] The population of *C. albicans* in feces was consistently higher in birds fed chlortetracycline- and oxytetracycline-supplemented diets than in birds fed a basal diet.[38] Chlortetracycline pretreatment definitely enhanced the toxicity of *C. albicans* in guinea pigs, rabbits, and monkeys.[31]

The theories that have evolved concerning this relationship were listed[11] as follows: direct enhancement of *C. albicans* growth; elimination of competitive flora; avitaminosis due to elimination of organisms that synthesize vitamins; and lowered resistance of host due to original infection, intensive therapy, or low nutritional state.

Prolonged oxytetracycline administration

The Sacramento laboratory noted in 1957 and 1958 that candidiasis was seemingly encountered characteristically following a prolonged course of antibacterial antibiotic medication.[8,9] This circumstance and the foregoing citations concerning this relationship served as stimuli for a trial.

Twenty 4-week-old White Leghorn (WL) (Kimber strain) cockerels, caged, were provided with unmedicated commercial feed, and with oxytetracycline solution at concentration of 400 mg/gal. as sole source of water. The aim was to determine whether this drug at the therapeutic level might incite endogenous *C. albicans* (assumed presence) into pathogenic action. The plan was to sacrifice individuals periodically for detailed examination. Chickens 1 and 2 were slightly listless on day 28, so were killed and examined. Both showed a slight degree of suspected intestinal candidiasis, but this was not confirmed by the cultural and histological means used. During the next 5 months, trial birds and controls were periodically sacrificed and examined, but no significant candidiasis was found by the diagnostic means used. Therefore, under the conditions of this trial, significant candidiasis was not incited by the drug during a 6-month administration period, and the trial birds at the end of the study manifested body condition comparable to that of the controls. Any conclusions must be guarded, however, because of questions of acquired resistance,[34] age resistance,[10] mild nature of

experimental infections,[43,44] and possible presence of infection in the interim periods (Underwood's[43] panendoscope would have been a diagnostic aid).

The disease in man and lower mammals

In man, according to Conant,[11] the most common involvements are of the mucous membranes and the skin. Bronchopulmonary involvement has been reported with increasing frequency ever since Castellani's important studies in 1927–28. Unusual involvements include: bone, joint, endocardial, meningeal, brain, corneal, and disseminated. Batista *et al.*[2] reported a significant association of *Candida* with chronic appendicitis in humans.

Kral[24] discussed the possibility that this disease may not be as rare in lower mammals as the scarcity of recorded cases indicate. Stuart[41] isolated *C. albicans* from an outbreak of acute mastitis and successfully reproduced the disease by inoculation with egg embryo culture.

Hinshaw's poultry isolates were studied by Wickerham and Rettger in 1939 and found to be indistinguishable from human-disease isolates.[17]

Therapeutic experiences

Copper sulfate (CS) in drinking water, at 1:2,000 dilution, was reported[15] somewhat promising as a treatment for candidiasis. Some found copper sulfate to be ineffective for this mycosis.[3,44] "Penotrane" (phenylmercuric dinaphthylmethane disulfonate) appeared to be fungicidal *in vitro*, for *C. albicans* at a dilution of 1:80,000 (poultry readily drank a 1:10,000 dilution),[18] Schaaf[35] regarded quaternary ammonium compounds, at dilution of 1:10,000, to be somewhat promising as a treatment. Nystatin (Mycostatin) was favorably reported[13,48,50] as a prophylactic or therapeutic treatment for candidiasis.

The task of appraising fairly the value of a given drug in a given field outbreak of candidiasis has been rather complex, more often than not, because of concurrent infections found in some outbreaks.[3,17,21] Therefore, as an effort in this direction, certain outbreaks were analyzed with a view to any complications present.

Premise SW (Sacramento County)

In the fall of 1959, a candidiasis outbreak occurred in a flock of 2,600 WL chickens, 9 months old, on wood shavings litter in a single house, divided into 3 pens, on a medium-large poultry operation. Sporadic occurrence of dark heads, diarrhea, and listlessness, with onset about a month previously, was gradually becoming more noticeable. Mortality had been low, but the egg production rate had gradually declined to about 40%. Oxytetracycline had been administered through the feed, at levels of 25, 150, and 200 grams per ton, on a non-definitive basis for the past month. Of 37 birds

examined, 16 had lymphomatosis but 20 had marked candidiasis; therefore a therapeutic trial was conducted with QAC (quaternary ammonium compound)[35] and nystatin.[42] The control pen was not an ideal control group, because it was the least-affected group initially, and during "cold snaps" its waterer was frozen less often than the others in the mornings. The pens under medication were affected by candidiasis to a similar degree. Prior to the 17th day of treatment egg production in the nystatin pen (B) and in the QAC pen (C) was comparatively "nip and tuck." However, on that day (2 days before discontinuance of medications) the former pen passed the latter, and later on it passed the control pen and clearly manifested a superior trend for the rest of the observation period of over 11 weeks.

Eventually, the above flock was marketed, the house thoroughly cleaned and disinfected, and a flock of replacement pullets placed in it. This new flock became diseased with candidiasis in September of 1960, following considerable fogging done within the house during a heat wave. Circumstantially, it appeared that the ground surrounding the house was heavily seeded during the 1959 outbreak, and dust storms and foot traffic served to recontaminate the house; or the fungus was resistant to disinfection efforts, as reported by some workers.[3,23] Eleven of 16 chickens (69%) examined in this outbreak had marked candidiasis. The simultaneous administration of QAC in water (1:10,000)[35] and nystatin in feed (100 grams per ton)[42] was apparently effective within pen A but was less effective in pen B. Pen C was clinically unaffected.

Premise JF (Placer County)

In May of 1958, an outbreak of candidiasis occurred in a flock of 7,800 turkeys, 6 weeks old (flock A), with an immediate past history of considerable therapeutic usage of chlortetracycline and oxytetracycline on a "blind" basis. All 4 birds submitted manifested severe candidiasis, with complications not evident. Copper sulfate in water (1:2,000)[15,16] was apparently efficacious.

In July of 1958, an explosive outbreak in a flock of 6,000 turkeys (flock B) had onset at 10 days of age. Of 75 poults examined, 56 had candidiasis (40 of them severe), 24 had coccidiosis (8% severe), 18 had *Escherichia coli* (O group 88) within fresh liver tissue, and 9 had air sac aspergillosis. The acute phase of the outbreak extended for about 1 month, and despite "heroic" efforts, over 3,000 had died and there were many stunted survivors. Copper sulfate (only medication tried for the fungal component), sulfaquinoxaline (SQ), erythromycin, chlortetracycline, and oxytetracycline were used singly and in various combinations. In retrospect, the *E. coli* finding may have had undue influence on the therapeutic approaches tried. The grower's claim that this brood had had a normal start in life was borne out by the fact that 87% of the 75 poults examined had evidence of normal yolk-sac absorption.

Premise GF (Placer County)

An outbreak had onset in mid-August of 1958 in a flock of 14,000 12-week-old turkeys. Of 27 birds examined, 22 showed candidiasis (moderate to severe) and 20 had coccidiosis (slight to moderate). Control efforts failed to prove effective, because the problems persisted recurrently for the rest of the grow-out period, which terminated with slaughter in mid-November, when numerous carcasses were graded down severely. In retrospect, the following cirucumstances appear as detrimental factors in the control program; the copper sulfate and sulfaquinoxaline were used alternately in water, despite the suggestion of simultaneous administration of SQ in feed and CS in water; the suggestion of increasing the pen area was ignored; the suggestion of improving water sanitation was slighted; and about 2 months after onset, the grower "traveled," returned wtih diagnosis of "infectious hepatitis," promptly abandoned the antifungal-coccidial approach, and adopted a high-level antibacterial antibiotic program. The therapeutic change promptly resulted in gross aggravation of the problem (daily mortality rate suddenly jumped from under 10 to over 20, and the general "tone" of the flock worsened).

Premise MB (Placer County)

During the first week of July, 1958, an outbreak occurred in a flock of 3,000 2-week-old turkeys. Of 17 poults examined, 16 had basically severe candidiasis and 6 had slight to moderate coccidiosis. The simultaneous administration of sulfaquinoxaline in feed and copper sulfate in water and certain sanitary improvements controlled this dual infection outbreak in a dramatic manner.

During the 2nd week of July, 1958, an outbreak occurred in a flock of 3,000 10-week-old turkeys. Of 12 turkeys examined, all had moderate to severe candidiasis, but 7 also had moderate coccidiosis and severe hexamitiasis. Simultaneous administration of sulfaquinoxaline in feed and alternate administration of copper sulfate and nithiazide in water arrested this complex outbreak satisfactorily.

SUMMARY

Poultry candidiasis within the Sacramento Valley, 1957-1960, was found in three adjoining counties in 1957 and in nine adjoining countries in 1960; was distinctly a dry season problem, with July being the peak month; in turkeys it occurred early in life (80% were in the first 3 months), and in chickens about 2/3 of the cases were in the 7-10 months' bracket. It was commonly associated with watering systems that were insanitary. It was more than occasionally encountered as a problem following a rather extensive course of anti-bacterial antibiotic medication and was basically an upper alimentary tract disease; possible intestinal and hepatic involvements are under study. Its course was favorably altered by

nystatin, quaternary ammonium compounds, and copper sulfate provided rather strict conditions for use were met.

Continuous oxytetracycline (therapeutic level) medication, which extended for over six months in some birds, failed to significantly incite endogenous *Candida* into pathogenic action, under conditions of a laboratory trial. Storage of the fungus in situ (portion of infected crop in covered Petri dish) at –20°C for 25 months did not have apparent lethal effect on the fungus. Isolates have produced classical, spectacular lesions and death in inoculated rabbits.

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ISOLATION OF AN ANAEROBIC VIBRIO FROM A KING PENGUIN (*APTENODYTES LONGIROSTRIS*)

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IN ADDITION to *Vibrio metchnikovi*, which is responsible for a cholera-like disease in chickens,[3] a number of other vibrios are known that produce disease in animals. These include *V. fetus*, from abortion in cattle and sheep,[7,11] *V. jejuni*, from diarrhea in cattle and calves,[6] and *V. coli*, from dysentery in swine.[2] An anaerobic species, *V. bubulus*, has been isolated from the reproductive tract of cattle but is probably not of pathological importance.[12]

In America a number of workers, including Delaplane *et al.*,[1] Moore,[9] and Winterfield *et al.*,[13] have described an

infectious agent associated with primary degeneration of the liver in poultry, while Peckham[10] and Hofstad *et al.*[5] have isolated an aerobic vibrio on solid media from field specimens of infectious avian hepatitis.

CASE REPORT

An adult King penguin (*Aptenodytes longirostris*) died suddenly after being in captivity for about 5 years. Post-mortem examination within 24 hours of the bird's death showed evidence of septicemia, but the immediate cause of death was internal hemorrhage from rupture of the liver capsule. Other abnormalities included hydropericardium, enlargement of the liver, which was a dark reddish-brown, and enlargement and pallor of both kidneys.

Avian vibriosis was suspected by the presence of numerous short Gram-negative spirals, consisting of two or three curved rods, in stained films of the heart blood and liver (Fig. 1).

METHODS

Material from various tissues was inoculated on a wide variety of media and incubated at 37 and 42°C in air, in an atmosphere containing 10% CO_2, and anaerobically.

Motility was demonstrated in the condensation water of 3-day-old glucose-agar slope cultures, and the test for indole

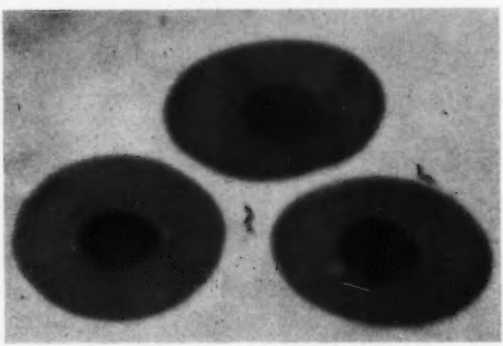

Fig. 1. Film of heart blood showing the typical "flying sea-gull" appearance of the organism. Giemsa. × 3,200.

was performed in 1-ml amounts of peptone water substituted for the condensation water of 48-hour glucose-agar cultures and reincubated for a further two days in the anaerobic jar. Three-day-old glucose-agar slope cultures were also tested for the production of hydrogen sulphide and catalase, by standard methods.[8]

The yolk sacs of eight-day-old embryonated eggs were inoculated with 0.1-ml suspensions in saline of penguin liver tissue that had been held at 5 days –28°C, and also with growth from a third-transfer blood-agar culture. The eggs were "candled" daily for six days, after which smears were prepared from the yolk sacs and the embryos examined for lesions. The strain's pathogenicity for laboratory animals was studied in pigeons, female guinea pigs, and white mice. The inocula were infected heart blood kept 3 days at 4°C, and a saline suspension of a 72-hour primary blood-agar culture. Doses of 0.2 ml were injected intraperitoneally or subcutaneously.

RESULTS

The presence of vibrio-like organisms in the penguin's

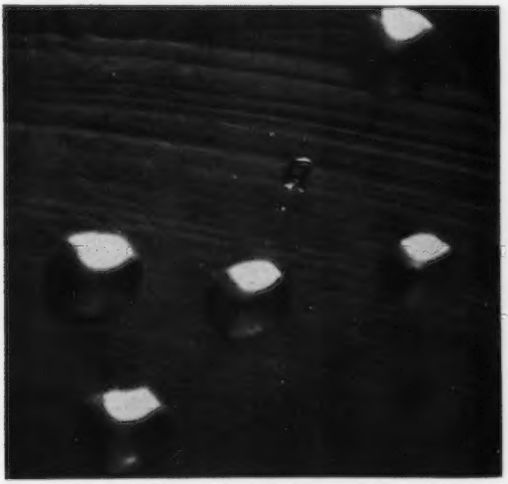

Fig. 2. Primary culture. Surface colonies (smooth form) on horse blood agar, 3 days, 37°C, anaerobically. × 15.

heart blood, liver, lungs, and kidneys was confirmed by examination of smears prepared from the slight surface growth observed on 48-hour-old blood-agar plates from the anaerobic jar only. All other cultures were apparently sterile except those from the lungs, which contained, besides vibrios, staphylococci and an unidentified *Haemophilus*. Compared with the primary cultures, the amount of growth became progressively poorer after each subculture and, although an attempt was made to find a more suitable medium, the strain died out after only four transfers.

Growth characters. Surface growth of the primary cultures on horse-blood agar showed tiny, punctiform, non-hemolytic, dew-drop colonies not exceeding 1 mm in diameter. They were smooth, entire, and low-convex, and emulsified easily (Fig. 2). After subcultivation, approximately equal numbers of smooth and rough colonies appeared, the former resembling those of the primary cultures whereas the latter were more opaque, drier, and less regular in outline (Fig. 3). After the second transfer, most of the colonies were of the rough variety, with a central raised portion and a bevelled periphery (Fig. 4).

On glucose-agar a delicate colorless growth was observed

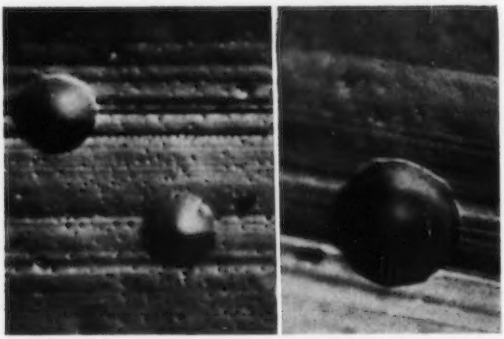

Fig. 3. First transfer. Surface colonies (intermediate form) on horse blood agar, 48 hours, 37°C, anaerobically. × 12.

Fig. 4. Third transfer. Surface colony (rough form) on horse blood agar, 3 days, 37°C, anaerobically. × 20.

at the edge of the slope between the agar and the glass container, and, after four days, tiny opaque colonies appeared as a narrow, raised line of growth at the interface of the condensation water and the surface of the agar.

There was no visible growth on various other media, including inspissated egg, Robertson's cooked-meat medium, and a modified Brewer's medium.[4]

The strain grew at 37°C, but not at 42°C, and then only under conditions of strict anaerobiasis. There was no evidence to suggest that it could be adapted to grow in air or in air containing 10% carbon dioxide.

Morphology. Primary cultures showed weakly staining Gram-negative comma-shaped rods, occurring singly or in pairs in the shape of an open ring resembling a U, and occasional short spirals (Fig. 5). Frequently present in stained films of subcultures were slender, straight rods, each with a tiny Gram-positive terminal granule, whereas older cultures consisted almost entirely of masses of Gram-negative pleomorphic coccal forms.

Motility. The strain was actively motile, its progress being

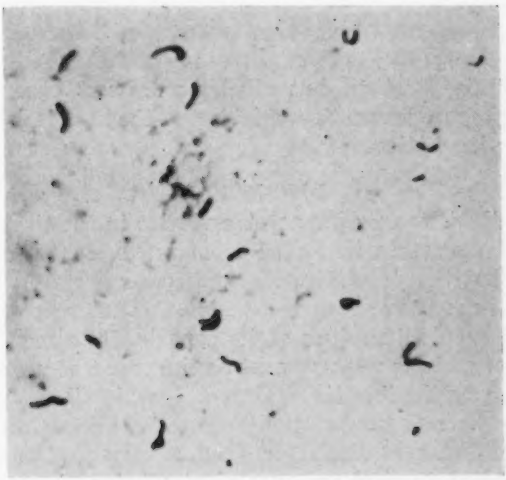

Fig. 5. From a horse blood agar culture, 3 days, 37°C, anaerobically, showing variation in morphology on artificial media. Gram. × 2,800.

characterized by alternate spinning and haphazard darting movements across the field.

Biochemical reactions. There was no evidence of indole, hydrogen sulphide, or catalase production by the methods used.

Sensitivity to antibiotics. With the disc technique, terramycin, chloromycetin, streptomycin, and penicillin gave large zones of inhibition of growth on blood-agar plates, but aureomycin was less effective and sulphafurazole completely failed to inhibit growth.

Growth in embryonated eggs. All the embryos were alive and healthy six days after inoculation, and smears of the yolk sac failed to show vibrio-like organisms.

Pathogenicity for laboratory animals. All the animals survived for 14 days, when they were destroyed. The guinea Pigs showed a slight congestion of the mucosa of the anterior uterine horns, but vibrios were not present in stained smears. The mice and one pigeon were apparently healthy. Examination of the other pigeon, which had been inoculated intraperitoneally, showed an unusual lesion of the liver surface, consisting of a pale, slightly depressed necrotic center, surrounded by a bright-red, raised edematous border.

Histologically, the liver showed numerous small areas of periportal infiltration, but vibrios were not observed and cultures prepared from the periphery of the liver lesion proved sterile on incubation.

DISCUSSION

The significance of anaerobic vibrios in the tissues of an adult King penguin that died from internal hemorrhage is obscure, but the presence of a septicemia suggests that the organisms may have been responsible for enlargement of the liver and eventual rupture of its capsule. It seemed unlikely that the organisms were present as the result of post-mortem invasion from the intestines, since anaerobic vibrios were not found in the intestinal contents of this bird or in fourteen other penguins that died later, from a variety of causes. Attempts failed to isolate anaerobic vibrios, by filtration

methods, from water samples taken from three different areas of the penguin colony.

Characterization of the strain was limited because of the failure to maintain it after the first few subcultures, but there was evidence that it closely resembled the anaerobic species *V. bubulus*, by forming non-hemolytic surface colonies on blood-agar medium, by failing to produce either catalase or indole, and by its inability to grow at 42°C and on bile-salt medium, urea medium, and citrate agar. The avian strain differed from *V. bubulus*, however, by failing to produce H_2S.

Although the post-mortem findings were not unlike those of avian infectious hepatitis, the penguin strain differed from other avian vibrios in that it was a strict anaerobe and grew on horse-blood agar without added chicken bile, failed to infect embryonated eggs, and was, apparently, non-pathogenic to pigeons and other laboratory animals.

ACKNOWLEDGMENT

Thanks are due Dr. A. Wilson Taylor for helpful criticism, and Mr. Richard Hood for photography.

SUMMARY

An anaerobic vibrio was isolated from the heart blood, liver, lungs, and kidneys of a King penguin. Its cultural and biochemical characteristics differed from those of other avian vibrios, and from the anaerobic species *V. bubulus*, but it was difficult to maintain for lack of a suitable medium, and died after the fourth transfer.

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REMOVAL OF AN INTRA-ABDOMINAL TUMOR OF A PIGEON BY RADICAL INCISION

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THE publication of a paper[2] on the surgical removal of subcutaneous tumors from parakeets has resulted in many interesting referrals. The technique has proved successful with canaries, finches, blue jays, ducks, geese, parrots, mynah birds, and several other exotic species. Approximately 10% of the tumor cases we see in birds are intra-abdominal neoplasms, usually malignancies involving the kidney or reproductive organs. Surgery on these has been uniformly disappointing owing to the inaccessibility of the tumors, and their large size before the bird is presented for treatment.

Radiation therapy has been successful in certain abdominal neoplasms[3] and parakeets are known to be quite resistant to total-body irradiation.[4] Nevertheless much work remains to be done in this field, and controlled studies with pre- and post-treatment biopsy and long-term follow-up should be

initiated to ascertain the full value of radiation therapy on the abdominal malignancies of birds.

In attempting surgery on intra-abdominal tumors it was felt that proper visualization might lead to greater success. The first opportunity to try a radical incision and complete visualization was presented in the following case.

A female homing pigeon was presented for initial examination by a local fancier who stated that she had been getting "heavy" for several weeks. Her abdominal wall was quite distended, and a large firm mass could be palpated in the abdomen, with several irregularly shaped smooth-sided nodules protruding from the main mass. Permission was granted to operate, though prognosis was guarded because we suspected a malignancy.

Equithesin has been found to be quite safe and effective as an anesthetic when given intravenously to the larger avian species if administered slowly to effect. When giving it intravenously the dose is about 1.0 cc per kg, or 50% of that used intramuscularly for parakeets, canaries, and other small birds.[1]

PROCEDURE

The bird weighed 495 g and was anesthetized with 0.6 cc of equithesin in the median vein, given slowly to effect.

The feathers were plucked from the entire abdomen, and the skin cleansed with pHisoHex (2,2′-methylenebis [3,4,6-trichlorophenol]) and water, followed by alcohol. The bird was draped, and sterile technique was used for the entire operation.

An initial incision was made on the midline from the keel to one centimenter anterior to the anal opening. At the midpoint of this incision an incision was made at right angles from one lateral extremity to the other, giving a cruciate incision that could be pealed in four directions. The entire tumor was inspected, including its dorsal attachment. It appeared to arise from the ovary, was reddish brown, $7.5 \times 5.0 \times 4.5$ cm, and had several smooth irregular nodules over its surface.

Histopathologically it was shown by examination at a later date that the great bulk of the mass was an organized blood clot. The main body of the tumor was a hemangioendothelioma. One of the tissue sections had large numbers of

blood-filled spaces lined with endothelium. The other section was almost devoid of blood cells but the presence of endothial cells was unmistakable, although they seemed to be arranged in papillary formation.

At this stage the bird's respiration became slow and shallow. Oxygen at 6 L per minute was administered by holding the oxygen tube at the external nares for the remainder of the operation. This materially assisted respiration.

A ligature of 000 medium chromic gut was placed around the base of the tumor, and the mass was removed. A small portion of the ovary was left intact since the bird's value was strictly as a breeding bird. Since gross inspection and midsaggital incision of the tumor (by an assistant) seemed to indicate this was not a malignancy, we felt justified in attempting to prolong the reproductive life of the bird by retaining some ovarian tissue.

Moderate hemorrhage was experienced but easily controlled with forceps and the topical application of thrombozyme (Pitman-Moore Co.) or pressure with gauze sponges.

The incision was sutured in two layers, the muscle with the peritoneum, and the skin. A simple continuous suture of 000 intestinal gut with swedged on atraumatic needle was used, starting at the four extremities of the incision and working toward the center, giving four separate suture lines in both the deep and superficial layers. Three hours postoperatively the bird was fully conscious and could stand, but experienced difficulty in walking. She was given 10 mg of tetracycline intramuscularly daily for five days. At the end of ten days the incision was completely healed by first intention, and the bird was able to walk and fly quite well.

As of this writing (11 months later) the bird is in excellent health, although attempts to use her for breeding have so far proved unsuccessful.

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ISOLATION OF A HEMAGGLUTINATING AGENT DISTINCT FROM NEWCASTLE DISEASE FROM THE RESPIRATORY TRACT OF CHICKENS

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This report describes investigation of an agent, isolated from the tracheas of three-week-old chickens with a respiratory disease tentatively diagnosed as infectious laryngotracheitis (ILT), that hemagglutinated chicken erythrocytes. Because of unusually high rate of spread and mortality, the case was referred to this laboratory for confirmation.[3]

HISTORY

A veterinarian described, by telephone, an unusual respiratory infection associated with a mortality of over 20% in a flock of fryers in the preceding 24 hours. The brood had been vaccinated when 4 days old with the B-1 strain of Newcastle disease (ND) vaccine given in the drinking water. No other vaccine was used. At 3 weeks old the flock became severely affected with a respiratory infection primarily involving the trachea. No other lesions were found on necropsy. Tentative diagnosis was ILT, and the tracheas of six birds were preserved in 50% glycerine and shipped to this laboratory for confirmation.

The tracheas were moderately congested with petechial hemorrhages in the mucosa. There was considerable caseous exudate in the larynx and in the upper third of the trachea. The exudates and curetted mucosa of the tracheas were ground with sterile alundum in buffered saline. A portion of the suspension was left untreated, and the other was treated with 5000 units of penicillin and 10 mg of streptomycin per ml

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and allowed to stand for 2 hr before being inoculated into chickens and embryonating eggs.

MATERIALS AND METHODS

Chickens. The chickens in these experiments, obtained as day-old chicks from a normal Poultry Husbandry flock of the University of California, were white Leghorns not vaccinated against any disease. The birds were raised in a house maintained specifically for raising normal birds for experiment, located about one-half mile from buildings and laboratories used for the study of poultry diseases.

Virus. Strain GB Texas, 1948, ND virus, was used for all challenges. The virus was propagated in embryonating chicken eggs, and the allantoic and amniotic (aa) fluids were pooled, distributed in 2-ml amounts, and stored at –6°C. The pool was titrated in embryonating chicken eggs at four-week intervals to determine constancy of titer. The challenge dose, given by the intramuscular route in all cases, was 2×10^5 embryo MLD_{50}. Birds were challenged with infectious bronchitis virus by inoculating intratracheally 0.2 ml of a 1:10 dilution of aa fluids of embryos previously infected with the Massachusetts strain of the agent. In all instances, susceptible control chickens were inoculated to test potency of the virus suspensions.

Antiserums. Chicken anti-ND serum was a pool, collected 25 days following recovery from a group of chickens vaccinated with the LaSota strain of ND virus and challenged intramuscularly with the GB strain of ND virus. The antiserum was found to neutralize at least 10^6 MLD_{50} of GB or Montana ND virus in embryonating eggs. The chicken and rabbit antiserums to the new agent reported herein were prepared by first inoculating rabbits intramuscularly and chickens intratracheally, and three weeks later inoculating intramuscularly with 0.5 ml of infected aa fluids four times at four-day intervals. Ten days after the last injection, the serums were collected from the individual animals, Seitz-filtered, dispensed in 2-ml ampoules, and stored at –6°C.

All other antiserums used were obtained through the courtesy of the following investigators: influenza A and B and mumps, from Dr. E. Lennette, California Public Health Laboratory, Berkeley, California; strains Brescia and N, fowl plague, from Dr. J. E. Williams, Animal Disease Eradica-

tion Division, Agricultural Research Service, Washington, D.C.; PPLO, from Dr. J. F. Crawley, Connaught Medical Research Laboratories, University of Toronto; the mycoplasma strain S6 from Dr. H. E. Adler, University of California, Davis, California; CELO, from Dr. V. J. Yates, University of Rhode Island, Kingston, Rhode Island. Antiserums of para-influenza 1 and 2 were purchased from Microbiological Associates, Bethesda, Maryland, and para-influenza 3, strains Bernard and Davis, from the 6th Army Medical Laboratory, Fort Baker, California.

Serology. Since the hemagglutination inhibition (HI) antibody to the newly isolated agent was low, the test, unless otherwise stated, was conducted in doubling dilutions using the beta procedure as described for the diagnosis of influenza[5]; however, the volumes consisted of 0.2 ml of virus titrated to contain 8 hemagglutinating (HA) units, with an equal volume of diluted suspected serum beginning with a dilution of 1:2. To this was added 0.4 ml of a 0.5% chicken red blood cell suspension. The hemadsorption and hemadsorption inhibition tests were conducted as previously described.[2]

Complement fixation tests. Complement fixation tests were conducted with Drs. R. W. McKinney and B. L. England at the 6th Army Medical Laboratory, Fort Baker, California. The test precedure was the modified Kolmer technique, described as the standard procedure for army installations.[5]

RESULTS

Reaction in susceptible chickens and embryonating eggs. Chickens inoculated intranasally and intratracheally with the treated or untreated suspension of tracheal exudates developed typical signs of laryngotracheitis beginning on the second day. The disease progressed rapidly, with a high mortality by the fifteenth day. Recovered chickens were immune to ILT 2–6 weeks later as determined by intrabursal instillation of ILT virus. Serums of recovered birds did not present agglutinins to mycoplasmas, HI or neutralizing (SN) antibodies to ND virus, or SN antibodies to infectious bronchitis virus. Repeated cultural examination revealed no *H. gallinarum* or *Mycoplasma gallisepticum.*

Antibiotic-treated tracheal exudate inoculated on the chorioallantoic membrane or into the allantoic sac of 8-day-old

embryonating eggs killed no embryos during a 6-day incubation period. However, embryos inoculated on the chorioallantoic membrane presented raised circular foci indistinguishable from ILT lesions with dwarfing and congestion of embryos in the first serial passage. Chickens inoculated intratracheally and intrabursally with a saline suspension prepared from the infected chorioallantoic membrane presented typical respiratory signs and bursal inflammatory reactions of ILT.

Passage of the tracheal exudate through embryos inoculated into the allantoic sac caused no death during a 6-day observation period during the first two passages; however, the aa fluids hemagglutinated chicken red blood cells. The third and fourth passages were respectively accompanied by a mortality of three of four embryos on the third passage, and four of four embryos on the fourth passage.

Treatment of the infected aa fluids with ND antiserums. The aa fluids of the third serial passage of the tracheal exudate through embryos were treated with known high-titer chicken anti-ND serum. Neutralization of the new agent was not evident following treatment of the infected aa fluids in three subsequent serial passages through embryos. Sporadic mortality in the inoculated embryos continued, and the aa fluids of the dead and surviving embryos agglutinated chicken red blood cells. Inoculation intratracheally or intrabursally into chickens produced typical reactions of ILT. Chickens inoculated intramuscularly were asymptomatic for 15 days, and their serums contained no HI or SN antibodies to ND or agglutinins for mycoplasma. All of the birds were susceptible to intramuscular challenge with a virulent GB strain of ND virus.

Treatment of infected aa fluids with ILT-immune serums. The aa fluids of the fourth serial passage of the original tracheal exudates and aa fluids of embryos of the following six generations were treated with an equal volume of chicken anti-ILT serum and incubated 3 hr at 4°C prior to inoculation. Hemagglutinins in the absence of lesions or death of the fifth, sixth, and seventh passages were found in all embryos after a 5-day incubation period. A greater mortality began with the

Table 1. Response of chickens inoculated with aa fluids of the tenth and eleventh passages through embryos of original tracheal exudates previously treated with ND and ILT antiserum.

Trial	No. of birds	Age (days)	Inoculum (aa fl)	Inoc. route	Signs	HI titer[b] to ND virus	HI titer[b] to MVY virus[b]	Challenge[a] ND virus	Challenge[a] ILT virus
1	4	37	10th pass.	1M	neg 21 da	all-0[c]	1-1/2 2-1/8 1-1/16	2/2	2/2
	4	37		IT	2 neg; 2 mild tranient, audible rales on 6th day[d]	all-0[c]	2-0 1-16 1-32	2/2	2/2
	2	37		IBurs	neg	all-0[c]	2-0		2/2
II	15	9	11th pass.	IM 0.2 ml	none	all-0[e]	—	15/15[e]	—
	5	9		Contact	none	all-0[e]	—	5/5[e]	—
	15	9		IT	none	all-0[e]	—	15/15[e]	—
	5	9		contact	none	all-0[e]	—	5/5[e]	—

[a] Suscept no. challenged.
[b] Serums tested 25 days following inoculation. Serums of trial I stored at -20°C and tested at a later date. Serums of trial II discarded, therefore could not be retested.
[c] Twenty-five days after inoculation.
[d] Agent was reisolated from trachea on fourth day from all four birds — pool of aa fluids of embryos labeled MVY agent.
[e] Four weeks after inoculation.

eighth serial passage, which in the tenth serial passage increased to 7 of 8 embryos inoculated.

Inoculation of chickens with the agent (Table 1). Inoculation of chickens 37 days old by the intramuscular and intrabursal routes with infected aa fluids previously treated with both ND- and ILT-immune serums (tenth embryo passage) resulted in no signs of a disease. Chickens inoculated intratracheally exhibited very mild, moist rales and abnormal chirps, unlike those observed with infectious bronchitis. The signs were detected on the sixth day in two of the four recipients. No other evidence of the disease was seen during the 21-day observation period. Tracheal swabs taken from all four of the chickens on the fourth day resulted in reisolation of a hemagglutinating agent in chicken embryos. The isolate was designated the MVY (myxovirus strain Yucaipa) agent for future experiment. On the twenty-fifth post-inoculation day none of the serums of the birds inhibited hemagglutination of red blood cells by ND virus (strain Montana), and the birds were found to be susceptible to challenge with ND and ILT viruses. The chicken serums, which were stored but later retested for HI antibodies to the MVY agent, presented specific antibody titers varying from 0 to 1:32.

No signs of respiratory disease were evident in a second trial with aa fluids of the eleventh serial passage inoculated intramuscularly and intratracheally into ND susceptible 9-day-old chicks. Four weeks after inoculation, all of the birds were susceptible to ND virus on intramuscular challenge.

Virulence for embryonating eggs. Table 2 shows the irregularity of death pattern and the presence of hemagglutinins in the aa fluids of 8-day-old chicken embryos inoculated wtih serially diluted suspensions of the MVY agent. Embryo mortality was highest with the higher concentrations of the agent; however, hemagglutinins were readily demonstrable in embryos that died and in most that survived for 12 days. Only seven embryos in the titration had no detectable hemagglutinins: one died 48 hours after inoculation with a 10^6 dilution of the infected fluid, and six embryos inoculated with the higher dilutions (10^8 and 10^9) were alive on the twelfth day. It is evident that, although the virus is not highly virulent for 8-day-old chicken embryos, the agent was highly

Table 2. Mortality and hemagglutinin titers in aa fluids of each of five 8-day-old embryos inoculated by the allantoic route with log dilutions of the MVY virus.

Log dilution of virus suspension[a]	Days post-inoculation[b]											
	Mortality										Survived	Total pos[c]
	2	3	4	5	6	7	8	9	10	11	12	
10^3	1 (1280)		1 (1280)	1 (320)	1 (1280)		1 (640)					5
10^4			1 (1280)		1 (1280)				2 (1-1280) (1-2560)		1 (1280)	5
10^5					4 (3-640) (1-1280)		1 (640)					5
10^6				1 (1280)	1 (1280)			1 (640)		1 (2560)	1 (>80)	5
10^7	1 (0)			1 (2560)			2 (1-640) (1-2560				1 (>2560)	4
				(640)							4 (2-0) (1-320) (1-640)	3
10^8				1							5 (4-0) (1-2560)	1
10^9												
Total	1 0	2	4	4	0	4	4	1	2	1	11	2

[a] Aa fluids of fourth serial passage of MVY virus in embryos.
[b] Including hemagglutination titers of aa fluids of each embryo.
[c] Embryos dead and hemagglutination titers of aa fluids of each embryo indicated in parentheses.

infectious for the embryonic tissues. Routine titrations of the MVY agent on serial passage through embryos consistently showed a 50% embryo infectivity titer of 5×10^8 per ml.

Inoculation of Mice. Infected aa fluids were inoculated by insufflation into 5 young mice. No evidence of infection of the respiratory tract or of a pneumonia was observed in 8 serial passages at weekly intervals.

Size of the agent. Filtration studies were made with infected aa fluids having a titer of 5×10^8 embryo infective doses (EID_{50}) per ml diluted 1:10 with brain-heart infusion broth. To this was added 2 ml of a culture of *Ps. aeruginosa*, and the mixture passed through a Seitz filter (EK) and serially through gradacol membranes to determine its size. The filtrates were tested for bacteria and virus by inoculation onto blood agar plates and into 9-day-old embryonating eggs (Table 3). The virus passed through the Seitz filter and through gradacol membranes of average pore diameters of 800 and 270 mμ but not of 140 mμ. From Elford's formula, the filterable virus appears to be larger than 70 mμ but smaller than 135 mμ, which is in the range reported for the viruses of the myxo virus group.

Thermostability of the agent. A group of 25 nine-day-old chicken embryonating eggs was inoculated with the MVY virus by the allantoic route. Only five (20%) of the embryos succumbed by the 120th hour. The aa fluids from the dead

Table 3. Filtration of fluids of embryos infected with the MVY virus through Seitz filter and graded gradacol membranes.

Passage no. of mixture	Filter[a]	APD[b]	Test of filtrate on agar and in embryos			
			Blood plate	Embryo inoculations		
				Mortality	Bact.	HA titer[c]
Original	None	—	+	5/5	+	80 or >
First	Seitz	EK	0	0/5	0	80 or >
First	Gradacol	800 mμ	+	5/5	+	80 or >
Second	"	270 mμ	0	0/5	0	80 or >
Third	"	140 mμ	0	0/5	0	0

[a] Gradacol membranes obtained from St. Mary's Hospital, London, England.

[b] Passed through gradacol membranes under 150 mm Hg pressure and through Seitz filters under 8 cm H_2O pressure.

[c] Reciprocal of titer.

embryos and from embryos that survived were pooled separately and titrated. The titer of each pool was 1×10^8 EID_{50} per 0.2 ml. One-ml portions of each pool were placed in Wasserman tubes and suspended in a 56°C water bath.

Survival of virus following exposure was determined by inoculating embryos with the fluids after 30, 60, 90, and 120 minutes. The virus of each pool withstood the temperature exposure over the longest period tested. The stability of the agent at low temperature was attested by its viability after 31 months at –6°C.

SEROLOGICAL STUDIES

Several tests were used for identification of the virus: the neutralization, hemagglutination, HI, complement fixation, hemadsorption, and hemadsorption inhibition tests. The tests were against all known agents commonly found in the respiratory tract of chickens, and a number of respiratory disease agents infectious to humans that hemagglutinate red blood cells.

Neutralization. Immune sera to a number of viruses were examined for their possible capacity to neutralize the agent. The neutralization tests were performed in 9-day-old chicken embryos using the allantoic cavity route for inoculation of the serum-virus mixtures. The virus was not neutralized by immune serums of the following viruses: influenza A, influenza B, mumps, ND virus (strain Montana), CELO, fowl plague, strains Brescia and N, Crawley and mycoplasma (S6).

Complement fixation. Antiserums produced in rabbits with the MVY virus specifically fixed complement in the presence of the infected aa fluids in dilutions of 1:32 to 1:64. No fixation of complement was demonstrated with known serums to the following viruses: mumps, sendai (para-influenza type 1), CA virus (para-influenza type 2), adeno virus type 4, and Bernard and Davis strains of hemagglutinating virus type 1 (para-influenza type 3).

Hemagglutination and hemagglutination inhibition. Although the agent hemagglutinated chicken red blood cells, the infectious aa fluids of embryos were tested for their capacity to hemagglutinate erythrocytes of chickens, guinea pigs, and human type-O blood. In the presence of chicken or guinea pig

erythrocytes, titers were slightly higher after 1 hr at 37°C than at room temperature (25–28°C). The hemagglutination titers were considerably lower when the tests were conducted at 4°C overnight. Virus hemagglutination was decidedly less stable with erythrocytes of human origin than with chicken or guinea pig erythrocytes. With human red blood cells a decided elution of virus was noted; the elution was more evident in tubes incubated at 37°C than at room temperature. Elution was more or less complete by 24 hours at 4°C.

Chickens inoculated intratracheally or intramuscularly with the MVY virus produced HI serum antibodies that specifically inhibited hemagglutination of chicken red blood cells by the homologous virus but not by ND virus. Conversely, ND antiserum did not inhibit hemagglutination of chicken red blood cells by the MVY virus. Fig. 1 illustrates the reactions to the MVY and ND viruses with serums of chickens recovered from the respiratory disease caused by the MVY

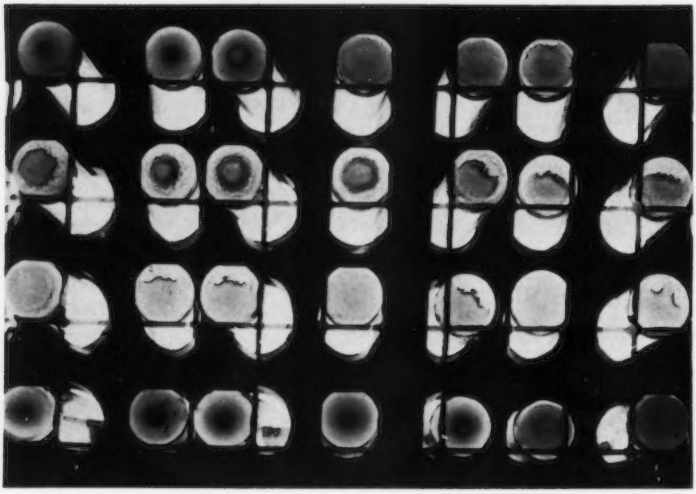

Fig. 1. Hemagglutination inhibition test using Newcastle disease virus and the MVY virus with serums of chickens recovered from both infections.

Bottom half: using 4 units of ND virus with chicken anti-ND (lower row) and anti-MVY (upper row) serums. *Upper half*: using 4 units of MVY virus with chicken anti-ND (lower row) and anti-MVY (upper row) serums.

Table 4. Hemagglutination by the MVY virus at various temperatures using chicken, guinea pig, and human type "O" erythrocytes.

Erythrocytes	Incubation	Dilution of virus—1 to: 4	8	16	32	64	128	256	512
Guinea pig	4°C overnight	++++	++++	++++	++++	+	—	—	—
	37°C 1 hr	++++	++++	++++	++++	++++	++++	+++	+
	RT[a] 1 hr	++++	++++	++++	+++	++	—	—	—
Chicken	4°C overnight	++++	++++	++++	+++	++	++	+	+
	37°C 1 hr	++++	++++	++++	++++	++++	++++	+++	++
	RT 1 hr	++++	++++	++++	++++	++++	++++	++	+
Human Type "0" cells	4°C overnight	—	+	—	+	+	+	—	—
	37°C 1 hr	++++	++++	++++	+++	+	—	—	—
	RT 1 hr	++++	++++	++++	++++	+++	+	—	—

RT = room temperature, 25–28°C.

virus and ND. No cross-hemagglutination inhibition was observed. It is evident, therefore, that the agent causing the mild respiratory disease was serologically as well as immunologically distinct from Newcastle disease virus (strain Montana).

In another experiment, HI antibodies appeared by the tenth day in the serums of a group of birds inoculated intratracheally with the MVY virus. The HI titers of the individuals varied considerably. In 26 birds inoculated intratracheally, 22 showed titers of 1:4 to 1:128 or greater by the thirteenth day. When the birds were re-bled two weeks later, titers decreased in one-half of the birds, but remained the same or increased slightly in the others. In some birds, HI antibodies were still detectable 68 days later.

Although erythrocytes from guinea pigs appeared very sensitive in the hemadsorption test (below), red blood cell suspensions from this species were compared with chicken

Table 5. Hemagglutination inhibition tests using chicken and guinea pig erythrocyte suspensions with serums of chickens and rabbits inoculated with the MVY virus.

	HI or non-specific HA titers[a]			
	Chicken rbc		Guinea pig rbc	
Serum source[b]	Non-specific[c] HA	HI	Non-specific[c] HA	HI
Ch	0	32	4	128
	0	32	4	128
	0	16	2	64
	0	16	4	128
	0	0	4	4
	0	0	4	8
	0	2	16	0
	0	0	2	4
	0	0	4	16
Ra hyper	0	32	8	128
	0	16	8	64
Ch hyper	0	32	32	256 or >
	0	0	32	0
Ch NDV hyper	0	0	0	0

[a] Titers expressed as reciprocal of dilution.
[b] Serums from chickens (Ch) or rabbits (Ra) following recovery or hyperimmunization (hyper) with the agent.
[c] Reciprocal of dilution at which complete or partial hemagglutination was observed.

erythrocytes for detecting antibodies in the HI test with serums of chickens and rabbits exposed to the MVY virus. The results (Table 5) showed that guinea pig erythrocytes hemagglutinated nonspecifically in the presence of most of the serums of chickens and rabbits. The nonspecific hemagglutination varied among the serum samples in dilutions of 1:2 to 1:32. Chicken and rabbit antiserums having HI antibodies to the agent when tested with guinea pig erythrocytes were detectable, as corroborated by the test conducted with chicken red blood cells. The titers with the latter cells, however, were lower by two or more doubling dilutions in almost every instance. No nonspecific hemagglutination was observed with the chicken red blood cells. It is evident that, when guinea pig erythrocytes are employed in the test, serums having a low titer may be masked and that each serum tested requires a titration to determine the extent of nonspecific hemagglutination. A greater disadvantage with the HI test using guinea pig cells was that the cells required 2–3 hr to settle in the tubes for a satisfactory reading.

Propagation of the MVY virus in tissue culture. The agent was found to be cytopathic for monolayers of pig kidney and HeLa cell tissue cultures. The cytopathic effect, however, was considerably milder than the destructive activity of the more virulent strains of Newcastle disease or an avirulent

Table 6. Relative degree of hemadsorption of erythrocytes of five species of animals in HeLa cell cultures inoculated 48 hours previously with the viruses of MVY virus, Newcastle disease (strain Montana), and type 1 myxovirus.

Virus[a]	CP[b] activity	Erythrocyte species[c]				
		Guinea pig	Chicken	Sheep	Horse	Bovine
Type 1 myxovirus	0	++++	—	∓	∓	++
MVY virus	0	++++	+++	+++	++++	++
ND (str Mont)	+	++++	++++	+++	++++	++++
None, control	0	0	0	0	0	0

[a] Each virus inoculated into 6 tubes.
[b] Visual inspection of cultures.
[c] 0.2 ml of a 0.4% rbc suspension and 0.8 ml of buffered saline added to each tube, which was previously washed 3 times with buffered saline. Reactions read after 30 minutes at room temperature.

tissue-culture-adapted strain.[1] The cytopathic activity was evident macroscopically by the 144th hour, and supernatant fluids of the cultures proved to be infectious for chicken embryonating eggs.

Hemadsorption tests. Since Vogel and Shelokov[9] illustrated that the viruses of the myxo virus group could be detected by hemadsorption of erythrocytes to cells infected in a tissue-culture system, this technique was tried with the MVY virus. Table 6 illustrates the relative degree of hemadsorption of erythrocytes of guinea pigs, chickens, sheep, horses, and cows in HeLa cell cultures inoculated 48 hours previously with the MVY virus, ND virus, and type 1 myxo virus. Although erythrocytes of all five species were hemadsorbed by the MVY and ND viruses, it was evident that guinea pig erythrocytes showed the greatest amount of hemadsorption activity with all three viruses.

Hemadsorption inhibition test. As another means of identification, antiserums of known potency of a number of viruses were tested for their inhibitory action on hemadsorption of guinea pig erythrocytes onto HeLa cell cultures infected with the MVY virus and the Montana strain of ND virus. Table 7 shows that the antiserums of influenza type A and type B, mumps, Crawley, mycoplasmas (S6), and normal chicken serums did not inhibit hemagglutination of tissue cultures infected with ND virus or the MVY agent; however, a specific inhibition of hemadsorption was demonstrated with the antiserums of ND virus and the MVY virus in the tubes inoculated

Table 7. Hemadsorption inhibition tests using a number of known antiserums in HeLa cell cultures inoculated 96 hours previously with viruses of ND (Montana strain) and the MVY virus.

Antiserums to:	Hemadsorption with guinea pig erythrocytes	
	ND virus	MVY virus
MVY virus	4+	0
Influenza, type A	4+	3+
Influenza, type B	4+	3+
Mumps	4+	3+
Newcastle Disease	0	3+
Mycoplasma (S6)	4+	3+
Crawley	4+	3+
Normal chick	4+	3+
None	4+	3+

with their homologous agent. No cross inhibition was observed between the viruses and the serums.

Since preliminary studies suggested that the MVY virus was not highly antigenic, hemadsorption inhibition tests were conducted with immune serums prepared in rabbits and chickens. The serums were employed undiluted, and diluted 1:2 and 1:10 in buffered saline. Undiluted serums inhibited hemadsorption of guinea pig cells, whereas serums from chickens with a low HI titer diluted 1:2 or more did not inhibit hemadsorption of the erythrocytes. Thus, antibody concentration is of considerable importance in conducting the test.

DISCUSSION

The observations presented are concerned with isolation of a virus from a flock of chickens with a severe respiratory infection and unusually high mortality. The virus appeared as a mixed infection with laryngotracheitis virus, which was readily detected by standard methods. The new agent, referred to as the MVY virus, was isolated from infected aa fluids treated with hyperimmune chicken anti-Newcastle disease and anti-laryngotracheitis serums.

The MVY agent was readily propagated in tissue cultures of HeLa and swine kidney cells and in chicken embryonating eggs inoculated into the allantoic sac or on the chorioallantoic membrane. Mortality in infected embryos was sporadic over a 12-day period; the aa fluids from the dead and the majority of the living embryos hemagglutinated red blood cells of chickens, guinea pigs, cattle, horses, and humans (type O), which were the only erythrocytes tested. The infectivity titer of aa fluids containing the agent for chicken embryonating eggs as determined by the presence of hemagglutinins in the aa fluids was consistently 1×10^8 per 0.2 ml of inoculum.

Intratracheal inoculation of chickens with infected aa fluids produced mild, moist audible rales between the fourth and the sixth days in most, but not all, of the birds exposed. The virus was readily isolated from the tracheae of infected chickens on the fourth day. Observations not recorded in this

report showed that the infection spread from affected to susceptible chickens housed in the same room.

The agent passed through a Seitz filter and, by passage through gradacol membranes, was found to have an average pore diameter between 70 and 135 mμ. The agent was viable after 31 months at –6°C, and after at least 120 minutes at 56°C.

Although no mortality followed intratracheal or intramuscular inoculation into chickens, their serums specifically inhibited hemagglutination of chicken erythrocytes with its homologous virus but not with Newcastle disease virus. Hemagglutination of red blood cells by reciprocal tests showed that anti-Newcastle disease serums did not inhibit the MVY virus. The new agent and Newcastle disease virus showed no cross hemagglutination reactions with serums of chickens recovered from both infections.

The combination of the biological and physical properties of the MVY virus (size, stability, pathogenicity for susceptible chickens and embryos, hemagglutinating red blood cells, and producing an HI antibody) readily eliminated the relationship to viruses of infectious bronchitis, ILT, fowl pox, Fahey-Crawley,[6,8] celo or quail bronchitis.[4] Chickens infected with the MVY virus were fully susceptible to the viruses of ILT, infectious bronchitis, ND, and fowl pox. Chickens immune to the four viruses were not challenged with the new agent, because distinguishing clinical infection in all instances was difficult.

Since hemagglutinating property is common to certain other viruses, neutralization tests were conducted to show that the agent was not related to fowl plague (strains Brescia and N), Newcastle disease (strains Texas, GB, LaSota, and Montana), mumps, and influenza types A, B, and C. In addition, the complement fixation test and HI tests conducted with known serums indicated that the MVY agent was not serologically related to the para-influenza types 1, 2, and 3. It is evident that the agent appears to be antigenically unrelated to the known pathogens usually encountered in poultry.

CONCLUSION

Although the significance of the respiratory disease agent isolated from chickens with a mixed respiratory infection was not evaluated entirely, it is evident that a virus immunologically and serologically distinct from other known avian viruses was isolated. The agent, which causes mild respiratory signs upon inoculation intratracheally, can be propagated in chicken embryos and tissue cultures. Infected fluids of embryos hemagglutinate erythrocytes and, upon inoculation into chickens, produce a specific HI antibody distinct from those of ND virus and other agents of the myxo virus group. The agent is, for the present, designated the myxovirus strain Yucaipa (MVY virus).

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PROCEDURE FOR MODIFIED COMPLEMENT FIXATION TEST WITH TURKEY, DUCK, AND CHICKEN SERUM ANTIBODY[a,b]

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If turkey, chicken, or duck antiserum is heated, it fails to fix guinea pig complement in the direct complement fixation test; if unheated, it fixes complement from guinea pigs to a certain extent. Heated turkey antiserum will fix guinea pig complement, however, if a small amount of fresh normal chicken serum is added to the immune system.[3] The normal factor in chicken serum that brings about this reaction was later found to be present in turkey and duck serum also. These three normal factors can be used interchangeably to produce the reaction, but quantity requirements vary. For better understanding of these reactions, the component parts of avian and guinea pig complement were studied.[2]

It has been known for some time[1,4,5,6] that each of the various parts of complement found in guinea pig serum has its function and that fixation takes place in a certain sequence. The kinetics was worked out by immune hemolysis, with the antigen being sheep erythrocyte, the antibody being hemolysin produced in rabbits, and the source of complement being

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guinea pig serum. The results of those investigations are shown in the following reaction:

Reaction:

$$E + A \longleftrightarrow EA$$

$$EA + C' \xrightarrow{Ca^{++}} EAC'1 \longrightarrow EAC'1,4$$

$$EAC'1,4 + C'2 \xrightarrow{Mg^{++}} EAC'1,4,2$$

$$EAC'1,4,2 + C'3 \longrightarrow E^* \quad \text{(ghosts-hemoglobin)}.$$

in which E = erythrocyte (antigen)
A = hemolysis (antibody)
C′ = whole guinea pig serum

C′1, C′2, C′3, and C′4 are components of complement.

C′1 and C′2 are heat-labile; C′3 and C′4 are relatively stable. All four must be present for immune hemolysis. EA is an immune complex made up of E, the sheep erythrocyte antigen, and A, the anti-sheep-cell rabbit antibody or hemolysin. In the presence of Ca ions this immune complex converts C′1 of guinea pig complement, which exists in whole fresh serum as a proenzyme, to an active enzyme, an esterase. This Ca-dependent enzyme (C′1), when activated, will inactivate, or "fix", C′4, and after this initial fixation the complex (EAC′1, 4) will fix C′2 in the presence of Mg^{++} ions. When this complex has formed (EAC′1,4,2), C′3 enters the reaction and causes hemolysis of the erythrocytes. This reaction has the advantage, for study purposes, of showing a visible change (hemolysis). It is obvious that if activation of C′1 failed there would be no hemolysis. When the reaction went to completion, hemolysis occurred and this proved that the rabbit antibody-antigen complexes had activated guinea pig C′1,

To determine whether avian antibody-antigen complexes could activate guinea pig complement and give a similar visible reaction, chicken hemolysin was prepared to replace rabbit. Chickens were immunized with sheep erythrocytes, and this antibody was used to sensitize sheep cells (EA ch). It was found that this avian complex could not activate guinea pig complement. There was no hemolysis of the avian-sensitized erythrocytes. The avian complex could activate chicken complement, however, giving hemolytic titers as high as 1/128 on certain individual sera.

This observation strongly suggested that immune complexes other than those in immune hemolysis might also fail to activate quinea pig complement. If this incompatibility existed between avian antibody and guinea pig complement, indications were that guinea pig C′1 had not been activated; therefore, the reaction leading to fixation had never been initiated. If a small amount of fresh normal chicken serum (ch C′1) was added, however, the chicken proenzyme was converted to the active form, and the reaction occurred. There appeared to be no incompatibility between the chicken enzyme and guinea pig C′4, 2, or 3. The conclusion that C′1 or cofactor of C′1 was involved in the reaction was reached by the process of elimination. The chicken factor was heat-labile, was found in midpiece (water-insoluble fraction) of complement, could be removed from the serum on kaolin, and was essential to immune hemolysis. C′2, although labile, was not found in midpiece. C′3 and C′4 were eliminated because they were relatively heat-stable. Because C′1 was labile, heated serum lacked avian C′1. This characteristic explained why fresh unheated antiserum containing C′1 fixed complement. During serum dilution procedure, however, the avian C′1 was diluted out. This was remedied by adding small aliquots (determined by titration) of fresh normal chicken serum to every antibody dilution, which provided the needed avian C′1. This made antibody the limiting factor instead of C′1.

The most satisfactory combination as a source of complement was chicken serum for C′1, and guinea pig serum for C′2, 3, and 4. Although turkey and duck C′1 were interchangeable with chicken C′1, the normal chicken serum (ch C′1) gave the best results (see Normal Serum). The complex for immune hemolysis was sheep cells sensitized with rabbit hemolysin. Investigation had shown chicken hemolysin to be much inferior to rabbit hemolysin, and since there was no incompatibility between rabbit antibody (hemolysin) and chicken C′1, it was adopted. Chicken serum was unsatisfactory as a source of complement, because the pools of serum were low in titer and had a tendency in low dilution toward nonspecific lysis.

All reagents and basic titrations are shown. For maximal titers, the order of addition of reagents must be observed:

Veronal-buffered saline stock solution

NaCl	85.0 g
Sodium 5,5-diethyl barbiturate (Merck)	3.75 g
$CaCl_2 \cdot 2H_2O$	0.220 g
$MgCl_2 \cdot 6H_2O$	1.015 g
5,5-diethyl barbituric acid (Merck)	5.75 g

Dissolve the acid in 500 ml of hot double-distilled water and add to solution of other components; cool and make up to 2,000 ml with water. Each day, dilute accurately 1 part up to 5 with water. The final pH of the diluted buffer should be 7.3–7.4 and should contain the divalent ions Ca^{++} and Mg^{++} in the order of $CaCl_2$, 0.00015*M*, and $MgCl_2$, 0.0005*M*. This stock solution should be refrigerated.

Modified Alsever's solution

Dextrose	2.05 g
Na citrate	0.9 g
NaCl	0.42 g
Citric acid	0.055 g
H_2O	100 ml

The Alsever's solution should be autoclaved 15 minutes at 15 lb and removed immediately. The solution should be clear and colorless.

Sheep erythrocytes. Whole blood is collected in Alsever's solution in a 1:1 proportion. It is mixed well to prevent clotting and stored at 4°C. A one-day supply of sheep erythrocytes is washed with veronal-buffered saline at least 3 times or until the supernate is clear. The cells should be centrifuged at the same speed and for the same length of time for each washing, usually 10 minutes at 1,000 rpm. These are considered packed cells, and dilutions are made by volume.

Hemolysin. Commercial anti-sheep hemolysin is very satisfactory. It has a high titer and remains stable for many months.

Complement. Guinea pig serum from a colony of at least 10 animals is pooled, cleared of cells, and immediately frozen in aliquots sufficient for one day's need. When the ornithosis system is tested, it is necessary to pre-test the serum of each guinea pig for antibody against the antigen before the serum is used as complement. It is not uncommon to encounter the antibody in fresh or lyophilized complement since guinea pigs

may have an inapparent infection caused by an organism belonging to this group of viruses.

Normal chicken serum (*N-ch*). Similarly to guinea pig complement, N-ch is collected, cleared of cells, and frozen in small aliquots as soon as possible. Because the normal factor is labile, the serum should not be refrozen after use. This must be tested for lack of antibody against the antigen used in the immune system. When N-ch is introduced into *turkey* or *duck* antibody-antigen complexes, the undiluted N-ch is used, but when the factor is introduced into *chicken* antibody-antigen complexes, the factor must be concentrated as follows:

Centrifuge serum to remove all particulate matter

Add 3 ml cold N-ch slowly to 30 ml cold 0.0005*M* KH_2PO_4 (pH approx 5.2)

Allow serum to precipitate 1 hour at 4°C

Centrifuge 15 min at 3,000 rpm

Discard supernatant

Take up precipitate in 1 ml N-ch previously heated 30 min at 56°C. Euglobulin dissolved in saline is anticomplementary, but loses this characteristic when dissolved in heated serum.

If preparation is cloudy, centrifuge lightly and discard precipitate.

Store at —20°C.

Table 1 shows titrations for optimal quantity of N-ch in

Table 1 (example). Titrations for optimum amount of normal chicken serum required to restore antibody titer of inactivated turkey serum.[a]

N-ch%.[b]	Test serum dilutions				Antigen control
	1/32	1/64	1/128	1/256	
0	0	0	0	0	0
2	4[c]	4	0	0	0
3	4	4	3	0	0
5	4	4	4	0	0
7	4	4	4	0	0
10	4	4	4	2	2

[a] Procedure for titration of antibody (Table 6 and 7).
[b] Calculated in relation to antigen aliquot.
[c] 4 = total inhibition of hemolysis; 0 = hemolysis; 2 and 3 = intermediate.

turkey or duck systems, or 3 times concentrated N-ch in chicken systems.

From one master set of serum dilutions, inactiviated 30 min at 56°C, multiple titrations of an immune serum are made with increasing amounts of unheated N-ch or 3 times concentrated N-ch in each set. Maximum titer requires a certain order of addition or reagents: 1) antibody, 2) N-ch, 3) antigen, and 4) guinea pig complement. Optimum is the least amount of N-ch that gives the maximal titer without causing the antigen control to become anticomplementary. The normal serum is dispensed from a 0.1- or 0.2-ml Kahn pipette. See Tables 6 and 7 for complete procedure of test serum titration.

While normal, chicken serum will adequately provide the avian C′1 for turkey and duck antibody. The amount of N-ch required is usually about 5% in relation to the antigen portion. Maximum titers of chicken antibody, however, require about 3 times this amount of N-ch. Certain pools of N-ch caused anticomplementary and lytic effects at this level. Having determined that the factor (N-ch C′1) was in the water-insoluble portion of the chicken serum (midpiece of C′1), attempts were made to fortify with this fraction. This proved to be impossible because of the anticomplementary effects of euglobulin. However, if this water-insoluble fraction was dissolved in heated normal serum (3:1 original serum euglobulin in heated normal chicken serum), this anticomplementary effect disappeared and the lytic effect was destroyed in heated serum. In this way the normal factor was concentrated and the objectionable aspects were removed.

To obtain maximum titers of *turkey antibody*, whole normal chicken serum was used in adequate amounts. Normal turkey was not quite as strong as normal chicken serum, and *normal duck serum* had to be concentrated.

To obtain maximum titers of *chicken antibody*, the normal factor in chicken, turkey, or duck serum was concentrated. Again, normal chicken serum seemed superior, although normal duck serum was satisfactory. Normal turkey had to be concentrated. In every case, the normal chicken factor was found most satisfactory.

Hemolysin titration (*Table* 2). The hemolysin titrations

are incubated 30 min at 37°C and read in terms of inhibition. The highest dilution showing a "sparkling clearness" is 1 unit. At least 2 units are used for sensitization. Because strong hemolysin cannot be added to unsensitized cells, the amount of hemolysin is calculated and diluted to a volume equal to the volume of 4% cells used. It is customary to make a subdilution of 1/100 hemolysin, which lasts a week or more. In the example in Table 2, assume that the titer is 1/7,000. Two units would be 1/3,500. It would take about .03 of 1/100 hemolysin to sensitize 1 ml of 4% cells.

Ex:	30 ml	4% cells
	X.03 ml	1/100 hemolysin
	.9 ml	1/100 hemolysin diluted to 30 ml

With the diluted hemolysin in one flask and an equal volume of 4% cells in another, pour quickly back and forth. This makes a 2% suspension sensitized with a little more than 2 units of hemolysin. The sensitized cells are ready for use after standing 15 min at room temperature.

Complement titrations (*Table 3*). The purpose of this modified type of complement titration is to guard against compounded anticomplementary effects. If Table 3 is compared with Table 7, it is seen that this titration is virtually a series of serum controls. The sera are tested at a dilution level of 1/8 with various dilutions of guinea pig complement. To put it another way, it is a complement titration in the presence of a 1/8 dilution of avian serum. This is the level used for screening sera in routine work. Unheated sera are used because, for screening purposes and routine titrations,

Table 2. Hemolysin titration (example).

Reagents	Hemolysin dilutions					Controls			Time
	1/1T[a]	1/3T	1/5T	1/7T	1/9T				
Hemolysin amt.	0.1	0.1	0.1	0.1	0.1	0.1	0	0	
Sheep cells 4%	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	30 min at 37°C
C'1/15 (excess)	0.2	0.2	0.2	0.2	0.2	0	0.2	0	
Diluent	0.4	0.4	0.4	0.4	0.4	0.6	0.5	0.7	
(Read in terms of inhibition)	0	0	0	0	2	4	4	4	

[a] Dilution of 1,000

Table 3. Complement titrations (modified for avian serum).

Reagents	Avian Serum number #1	#2 etc.	Time
Step I			
Serum dilution 1/8	.2	.2	
C′ 1/22, 1/24, or 1/26	.1	.1	1 hr. at 37°C
Diluent	.3	.3	
Step II			
Sensitized cells	.2		30 min at 37°C

Readings will vary according to dilution of C′ used.

the sera need not be heated unless they tend to be anticomplementary. The complement must be tested at various levels because of seasonal changes in activity.

Use any 10 unheated immune or normal avian sera diluted 1/8. Mix at least three dilutions of complement, 1/24, 1/26, 1/28. Test each of the sera separately with each of the respective complement dilutions. After 1 hr at 37°C, add sensitized cells. The highest dilution of C′ contained in 0.1 ml causing lysis of the cells is optimum. This is called a half portion (0.1 ml usually contained about 2 units of complement by standard titration) and is used in the antigen as well as serum controls. The tests (antibody, N-ch, antigen, and complement) receive 0.2 ml or full portions. The use of half portions in controls minimizes the danger of mistaking compounded anticomplementary effects for specific fixation. Al-

Table 4. Antigen titration with turkey antiserum (example).

Reagents	Antigen dilutions 1/8	1/16	1/32	1/64	1/128	Ab control	Time
Step I							
Ag amt.	0.2	0.2	0.2	0.2	0.2	0	1 hr at 37°C
N-ch (approx. 5%[a])	0.01	0.01	0.01	0.01	0.01	0	
Ab 4 units	0.2	0.2	0.2	0.2	0.2	0.2	
C′gp	0.2	0.2	0.2	0.2	0.2	0.1	
Diluent	0	0	0	0	0	0.3	
Step II							
Sensitized cells 2%	0.2	0.2	0.2	0.2	0.2	0.2	30 min at 37°C
Readings	4	4	4	4	3	0	

Key: Ag = antigen; Ab = antibody serum; N-ch = unheated normal chicken serum.

[a] 5% in relation to antigen aliquot.

though complement controls are included each day, the complement is titrated only after each fresh bleeding. It is stored at –20°C.

Antigen titration with turkey antiserum (Tables 4 and 5). The titrations in Tables 4–7 are examples of turkey antibody-antigen complexes. Avian serum *must* be used as antibody in antigen titration. *For full titer the reagents must be dispensed in the order shown.* Normal undiluted, unheated chicken serum is used with turkey or duck antiserum; 3× concentrated preparation described under Normal Serum is used with chicken antiserum. The amount of N-ch is dispensed from a 0.1- or 0.2-ml Kahn pipette.

Complement fixation is a two-step reaction involving two immune systems. In the first step there is a mixture of antigen, antibody, and complement (in Tables 4–8, complement consists of both chicken and guinea pig serum the strength of which has been determined by titrations in Tables 1 and 3). This mixture is incubated 1 hr at 37°C. If both antigen and antibody are present, a complex will be formed that will "fix" or inactivate the complement. If either one is absent, the complement remains free. Because these reactions are not visible, a second known immune complex is added. This consists of sheep cells (antigen) sensitized with hemolysin (antibody). If there was an antigen-antibody complex present in step 1, the complement will have been fixed. This results in no change of appearance to the sensitized cells added in step 2 (inhibition or positive reaction). However, if either antigen or

Table 5. Antigen control (example).

Reagents	Antigen dilutions 1/8	1/16	1/32	1/64	1/128	Time
Step I						
Ag. amt.	0.2	0.2	0.2	0.2	0.2	
N-ch	0.01	0.01	0.01	0.01	0.01	1 hr at 37°C
C′	0.1	0.1	0.1	0.1	0.1	
Diluent	0.3	0.3	0.3	0.3	0.3	-
Step II						
Sensitized cells 2%	0.2	0.2	0.2	0.2	0.2	30 min at 37°C
Reading	0	0	0	0	0	

Table 6. Antibody titration (example).

Reagents	Antibody dilutions 1/8	1/16	1/32	1/64	1/128	Ag control	Time
Step I							
Ab amt.	0.2	0.2	0.2	0.2	0.2	0	1 hr at 37°C
N-ch (approx. 5%)	0.01	0.01	0.01	0.01	0.01	0.01	
Ag - 2 units	0.2	0.2	0.2	0.2	0.2	0.2	
C′	0.2	0.2	0.2	0.2	0.2	0.1	
Diluent	0	0	0	0	0	0.3	
Step II							
Sensitized cells 2%	0.2	0.2	0.2	0.2	0.2	0.2	30 min at 37°C
Reading	4	4	4	4	1	0	

Table 7. Antibody control (example).

Reagents	Antibody dilutions 1/8	1/16	1/32	1/64	1/128	Time
Step I						
Ab amt.	0.2	0.2	0.2	0.2	0.2	1 hr at 37°C
C′	0.1	0.1	0.1	0.1	0.1	
Diluent	0.3	0.3	0.3	0.3	0.3	
Step II						
Sensitized cells 2%	0.2	0.2	0.2	0.2	0.2	30 min at 37° C
Reading	0	0	0	0	0	

antibody is absent, the complement will cause hemolysis of the sensitized cells during the 30-minute period of step 2 (negative reaction). In the examples given in Table 4, 30 minutes after addition of sensitized cells, there was total inhibition of all dilutions up to 1/64. This is called one unit of antigen. Two units will be 1/32, etc. However, the serum control (Table 4) and all antigen controls (Table 5) hemolyzed because in each of these controls either antigen or antibody was absent. Had they failed to hemolyze, it would indicate a nonspecific inactivation of complement. No reagent can be used at a level that shows this reaction (anticomplementary).

Antibody titration (*Tables 6 and 7*). The antiserum can be tested unheated or after heating 30 min at 56°C. The same two-step reaction occurs in antibody titrations, and again the highest dilution causing total inhibition of the indicator system is one unit (Table 6) or the "titer" of serum. The antigen control (Table 6) and serum controls (Table 7) are hemolyzed, indicating that neither the antigen nor the serum was anticomplementary.

Daily controls (*Table 8*). The controls in Table 8 are essential to prove that all reagents are present in adequate amounts. These tests should precede the testing of avian serum. The first control is a serum known to contain antibody. As suggested in the table, tube 1 demonstrates the presence of fixation of complement by antibody in the presence of antigen. Tube 2, without the antigen, shows no fixation. This proves that the test antiserum is not anticomplementary (no nonspecific fixation).

The serum in tubes 3 and 4 lacks antibody (normal serum), and therefore shows no fixation (hemolysis). Tube 3 also shows that there is no nonspecific reaction between antigen and normal heated avian serum.

Tubes 5 and 6 are in the nature of a short titration for adequate amount of antigen. Both tubes show inhibition, which demonstrates the presence of 2 units or more of antigen.

Tubes 7, 8, and 9 are a short titration for units of complement. It has been found by the conventional method of titration that 4 units is usually equivalent to a "full portion." The above *modified type* of titration (Table 3), however, is

Table 8. Daily controls.

Tube arrangement: (1) (2) (3) (4) (5) (6) (7) (8) (9)		
Protocol	*Readings*	
Tube (1) Known positive antiserum (4 units) N-ch Antigen C′ (full portion)	4+	inhibition
Tube (2) Known positive antiserum control C′ (½ portion)	0	inhibition
Tube (3) Known negative serum N-ch Antigen C′ (full portion)	0	inhibition
Tube (4) Known negative serum control C′ (½ portion)	0	inhibition
Tube (5) .2 ml antigen (minmum 2 units) .01 ml N-ch .2 positive antibody (4 units) .2 C′ (full portion)	4+	inhibition
Tube (6) Same as (5) except .1 ml antigen (minimum 1 unit)	4+	inhibition
Tube (7), (8), and (9) C′ control, 0.1, .05, and .025; usually respectively 2, 1 and ½ units by standard C′ titration.		

more valuable for detecting anticomplementary effects resulting from aging serum or from seasonal changes. Tubes 7 and 8, respectively containing 2 and 1 units of complement, show complete hemolysis, whereas tube 9, containing a half unit of complement, shows partial hemolysis. All tubes are made up to 0.6 ml with diluent. The timing and hemolytic indicator system are those used for antibody titration. After assurance that the immune system is operating satisfactorily, unknown sera are screened at a 1/8 dilution. The quantities of the reagents are shown under Antibody Titration (Tables 6 and 7).

DISCUSSION

With an understanding of the mechanism involved in complement fixation, it becomes evident that the inability of heated avian serum to fix guinea pig complement stems from a failure of avian antibody-antigen complexes to activate guinea pig C′1. With addition of chicken C′1 (N-ch) to the system, however, the avian complex initiates the conversion of the avian proenzyme to the active form, and the reaction goes to completion.

The variation among the species in the quantity of normal factor required has not been explained. It is difficult to reconcile the requirements of increased amounts of normal factor for the titration of chicken antibody. If the amount of normal chicken factor required for titration of turkey and duck antibody were also adequate for chicken, it might be assumed that the relations were qualitative. However, titrations of the homologous chicken antibody required three times as much of the chicken factor. When the chicken C′1 was purified by continuous-flow electrophoresis and later scanned, the fraction appeared to have two parts.[2] This was interpreted as meaning either that the fraction contained C′1 and a cofactor or that C′1 is not a single substance. Attempts will be made to split fraction by repeated purification.

CONCLUSION

There is an incompatibility between certain avian antibody-antigen complexes and C′1 of guinea pig complement. Heated turkey, duck, or chicken antibody can be measured by adding avian C′1, or C′1 and cofactor to the immune system. A technique is offered.

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THE DEVELOPMENT OF RESISTANCE TO SPIRAMYCIN, STREPTOMYCIN AND CHLORTETRACYCLINE BY *MYCOPLASMA GALLISEPTICUM* IN CHICK EMBRYOS

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The phenomenon of bacterial resistance to drugs poses one of the most important questons in the investigations of a new drug for chemotherapeutic use: How rapidly will resistance of virulent organisms appear? We have attempted to answer that question concerning three drugs for the pleuropneumonia-like organism *Mycoplasma gallisepticum*, an etiological agent of chronic respiratory disease in chickens, by inoculating the organism into chick embryos containing an amount of the drug calculated to save about 50% of the embryos. We then harvested tissues from the embryos that died, reasoning that this would be the best source of antibiotic-resistant virulent *Mycoplasma gallisepticum*. Five serial passages were made, each time using as inoculum the tissue harvested from the previous passage. Then the drug was titrated against the culture to see if the culture had become resistant.

MATERIALS AND METHODS

Seven-day-old embryos in eggs from PPLO-free White Leghorn hens were infected by injecting into each yolk sac 0.2 ml of a brain-heart infusion broth (product of Digestive Ferments Co.; dilution usually 1:100) of a yolk sac suspension from embryos that died seven days after infection with *M. gallisepticum*. (Embryos dying on earlier or later days were discarded since harvesting at a uniform time after infection was found to maintain a constant level of virulence.)

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The organism used was a strain that has been passed in chick embryos for several years and has been characterized by Dr. Julius Fabricant as S-6 serological type.

About a half-hour before infection, the drug being studied was injected into the allantoic cavity of the embryos in 0.2 ml of distilled water. Groups of 20–70 embryos were used in order to ensure having embryos dead at the proper time for passage.

After a series of five passages the drug was titrated simultaneously against the parent strain and the passage strain to see whether the median effective dose (ED_{50})[3] of the passage strain was significantly greater than that of the parent strain.

The drugs used were streptomycin sulfate, spiramycin base, and chlortetracycline hydrochloride.

RESULTS

Streptomycin. In the first series of passages, which was preliminary in nature, each embryo was injected with 2 mg of streptomycin and infected with 0.2 ml of a 10^{4} dilution of yolk sac suspension. Percentage of survivors was high for the first three passages, and then dropped abruptly. After three more passages the dose of streptomycin was increased to 4 mg per embryo, and the infecting dose to 0.2 ml of a 10^{-3} dilution of yolk sac suspension. After four more passages the parent and resistant strains were compared for sensitivity to streptomycin. The dose-response curve of the parent strain was very flat, but the ED_{50} could be estimated at 4.3 mg per embryo. Only one of a group of 37 embryos infected with a similar dose of the resistant strain was saved by 16 mg of streptomycin per embryo.

The virulence of the two strains was determined by titrating them in embryos with and without streptomycin. Development of resistance to streptomycin had not diminished virulence, as shown in Table 1.

A second series of 10 passages was done, using a 4-mg dose of streptomycin and 0.2 ml of a 10^{-3} dilution of yolk sac as the infecting dose. The 4-mg dose of streptomycin did not

Table 1. Virulence titration of streptomycin-resistant and parent strains of *M. gallisepticum* in 7-day-old chick embryos; survival at 11 days.

	Streptomycin-resistant						Parent					
	Treated[a]			Untreated			Treated[a]			Untreated		
Dilution	Alive total	% alive	MST[b] (days)	Alive total	% alive	MST (days)	Alive total	% alive	MST (days)	Alive total	% alive	MST (days)
10^{-3}	0/70	0	6.6	0/76	0	6.8	26/61	43	7.3	3/73	4.1	6.3
10^{-4}	0/68	0	6.9	3/73	4.1	7.2	42/64	66	7.9	8/74	11	6.7
10^{-5}	0/70	0	7.1	3/71	4.2	7.8	—	—	—	4/69	5.8	6.8
10^{-6}	5/68	7.3	7.2	7/71	9.9	8.4	—	—	—	3/62	4.8	7.6
10^{-7}	13/72	18	8.4	24/76	32	8.9	—	—	—	20/66	30	8.2

[a] 16 mg of streptomycin per embryo.
[b] MST = mean survival time.

save many embryos from such a large infecting dose, as shown in Table 2. A titration was done after five passages, but the dose-response curve was so flat that a meaningful ED_{50} could not be calculated. It was clear, however, that some resistance had developed. Another titration was done after five more passages. The ED_{50} of streptomycin for the parent strain was 4.5 mg per embryo, but groups infected with the resistant strain had only 15–25% survivors at doses up to 16 mg per embryo.

Spiramycin and chlortetracycline. The culture of *M. gallisepticum* was tested for ability to develop resistance to spiramycin or chlortetracycline by injecting it into embryos containing 0.5 mg of chlortetracycline or 0.05 mg of spiramycin per embryo. A third series of passages was made in untreated groups. The results are in Table 3. At the end of the fifth, tenth, and fifteenth passages, each strain was titrated against the antibiotic with which it had been run. The parent strain was tested concurrently against both antibiotics. At the end of the fifth and tenth passages, there was some slight suggestion of increased resistance to chlortetracycline, so after the fifteenth passage a very careful titration was done. The results (Table 4) show that no resistance had developed.

Table 5 shows the results of the comparable titrations

Table 2. Development of resistance to streptomycin by *M. gallisepticum*[a] in chick embryos (2nd series); survival at 11 days.

	Streptomycin-treated[b]			Parent		
Passage	Alive total	% alive	MST[c] (days)	Alive total	% alive	MST (days)
1	9/42	21	9.7	1/49	2	6.2
2	8/67	12	9.8	0/69	0	6.2
3	10/67	15	9.9	4/67	6	6.2
4	6/65	9	10.0	0/67	0	6.6
5	4/67	6	9.3	0/48	0	6.5
6	10/68	15	9.4	0/66	0	6.9
7	10/66	15	9.4	4/66	6	6.8
8	13/66	20	9.0	7/67	10	6.8
9	5/69	7	8.8	3/56	5	6.9
10	2/68	3	8.8	0/68	0	6.9

[a] The embryos were infected with 0.2 ml. of a 10^{-3} dilution of a yolk-sac suspension.
[b] 4 mg of streptomycin/embryo.
[c] MST = mean survival time.

Table 3. Development of resistance to chlortetracycline and spiramycin by *M. gallisepticum* in chick embryos; survival at 11 days

Passage	Chlortetracyline[a]			Spiramycin[b]			Parent		
	Alive total	% alive	MST[c] (days)	Alive total	% alive	MST (days)	Alive total	% alive	MST (days)
1	10/67	15	9.0	6/69	9	8.4	0/9	0	7.9
2	15/67	22	8.8	9/67	13	9.0	3/68	4	6.9
3	26/67	39	9·2	12/67	18	9.1	0/69	0	7.4
4	17/52	33	7.8	2/63	3	8.3	0/65	0	6.0
5	26/64	41	9.0	11/66	17	9.1	0/68	0	7.8
6	18/63	29	7.5	8/68	12	9.1	0/65	0	6.5
7	23/61	38	8.0	7/65	11	8.4	0/67	0	6.1
8	5/59	8	8.4	2/68	3	8·4	0/68	0	6.2
9	39/59	66	9.2	0/66	0	8.7	0/65	0	6.5
10	16/60	27	8.1	4/69	6	8.0	0/65	0	6.4
11	18/43	42	8.0	3/48	6	8.7	1/49	2	6.2
12	19/56	34	8·2	5/67	7	9.5	0/69	0	6.2
13	35/57	61	8.6	3/67	4	7·4	4/67	6	6.2
14	18/66	27	8.1	0/65	0	7.8	0/67	0	6.6
15	22/60	37	8.3	1/66	2	7.5	0/48	0	6.5

[a] 0.5 mg/embryo, except passage 2, which had 0.25 mg/embryo.
[b] 0.05 mg/embryo.
[c] Mean survival time of embryos that died. All embryos were infected with 0.2 ml of a 10^{-3} dilution of yolk sac suspension.

Table 4. A comparison of strains of *M. gallisepticum* passed through chick embryos with and without 0.5 mg of chlortetracycline per embryo; survival at 11 days.

No. of passages	Dose (mg/egg)	With chlortetracycline			Without chlortetracycline		
		Alive total	% alive	MST[a] (days)	Alive total	% alive	MST (days)
5	2	3/8	38	6.4	3/7	43	8.0
	1	2/6	33	5.0	2/7	29	7.6
	0.5	5/10	50	7.2	2/8	25	9.8
	0.25	0/8	0	9.5	4/9	44	8.4
	0.125	0/9	0	7.8	0/9	0	8.6
	None	0/10	0	7.3	0/10	0	7.3
10	2	2/6	33	6.0	5/6	83	9.0
	1	6/10	60	9.5	2/7	29	10.0
	0.5	0/9	0	9.2	2/8	25	9.7
	0.25	0/9	0	7.9	4/10	40	8.7
	0.125	0/8	0	7.0	1/8	13	7.9
	None	0/10	0	6.7	0/10	0	7.0
15[b]	2	22/28	79	7.1	12/17	70	8.5
	1	11/29	38	9.0	17/29	59	8.7
	0.5	7/37	19	8.3	15/36	42	9.1
	0.25	7/40	18	8.7	5/34	15	8.7
	0.125	—	—	—	3/39	7.7	8.7
	None	3/40	7.5	8.1	3/37	8.1	7.5

[a] MST = mean survival time.

[b] Median effective dose with 95% confidence limits: with chlortetracycline, 0.93 (0.65-1.34) mg/embryo; without chlortetracycline, 0.81 (0.63-1.04) mg/embryo.

Table 5. A comparison of strains of *M. gallisepticum* passed through chick embryos with and without 0.05 mg of spiramycin per embryo; survival at 11 days.

No. of passages	Dose (mg/egg)	With spiramycin			Without spiramycin		
		Alive total	% alive	MST[a] (days)	Alive total	% alive	MST (days)
5	0.2	5/9	56	9.5	7/9	79	9.0
	0.1	6/10	60	8.2	6/9	67	10.3
	0.05	2/10	20	10.2	1/10	10	9.4
	0.025	1/10	10	9.2	1/10	10	8.2
	0.0125	1/9	11	8.9	0/10	0	8.0
	None	0/7	0	7.9	0/10	0	7.3
10	0.4	3/10	30	9.9	8/9	89	10.0
	0.2	2/7	29	8.8	5/10	50	10.6
	0.1	0/10	0	9.6	0/10	0	9.3
	0.05	0/9	0	8.7	2/9	22	9.9
	0.025	0/10	0	8.2	0/10	0	8.9
	None	0/10	0	7.1	0/10	0	7.0
15[b]	1.6	1/35	3	8.5	—	—	—
	0.8	1/36	3	8.5	—	—	—
	0.4	0/34	0	8.3	33/35	94	7.0
	0.2	1/39	3	7.7	19/37	51	10.4
	0.1	1/40	3	8.4	5/38	13	9.7
	0.05	—	—	—	6/37	16	9.6
	None	2/39	5.1	8.0	3/37	8.1	7.5

[a] MST = mean survival time.
[b] Median effective dose with 95% confidence limits, 0.18 mg/embryo (0.16-0.20).

with spiramycin. Resistance to this antibiotic was clearly demonstrated. At the end of five passages there was no evidence of resistance, at the end of ten passages it seemed likely that some resistance had occurred but after the fifteenth passage there had been at least a tenfold increase in resistance since the ED_{50} for the parent strain was 0.18 mg per embryo and less than 50% of the embryos infected with the resistant strain survived even on the dose of 1.6 mg per embryo.

DISCUSSION

The occurrence of resistance to streptomycin by *Mycoplasma gallisepticum* has been reported from the field,[1] and these resistant strains were considered to be virulent. We have shown by this technique that resistance can develop very rapidly in the laboratory and that virulence is maintained.

Spiramycin, which we have previously reported to be very effective in controlling experimental *Mycoplasma gallisepticum* infections in chick embryos[5] and in chicks[2] is also unsatisfactory since at least a tenfold resistance developed in less than 15 passages. We are not aware of any field use of spiramycin.

Resistance to chlortetracycline did not emerge during 15 passages or over a period of 15 weeks in which the organism was constantly in the presence of an amount that prevented mortality of about 50% of the embryos. We know of no other attempt to show occurrence of resistance by *Mycoplasma gallisepticum* to chlortetracycline experimentally. However, Osborn *et al.*[4] studied the development of resistance to antibiotics by *Mycoplasma* in turkeys with naturally occurring infectious sinusitis. In an experiment in which drugs were administered over a period of five months they showed that resistance to dihydrostreptomycin, chlortetracycline, oxytetracycline and tetracycline increased significantly when measured *in vitro*. Resistance to erythromycin did not increase, but the initial minimum inhibitory concentration was 100 mg/ml. Our experience with erythromycin in chick embryos[5] and that reported by Yamamoto and Adler[6] *in vitro* and *in vivo* suggest that the strain referred to above was so resistant on

first isolation that an increase in resistance would not be expected. No evidence for virulence of the resistant isolates was mentioned but antibiotic-resistant isolates from poults hatched from eggs from the infected turkeys were shown to be of very low virulence. In the work reported in short-term therapy, no resistance occurred to the tetracyclines or erythromycin but a resistant virulent strain of PPLO was isolated after a single injection of dihydrostreptomycin.

SUMMARY AND CONCLUSIONS

1. Resistance to streptomycin developed rapidly (in about five passages) in a strain of *M. gallisepticum* grown in chick embryos in the presence of the antibiotic.
2. The increased resistance to streptomycin was not accompanied by a loss of virulence.
3. Resistance to spiramycin developed about as quickly as that to streptomycin. In both cases resistance was complete at the maximum dose tolerated by chick embryos.
4. Resistance to chlortetracycline did not occur in fifteen passages through embryos treated with the antibiotic.

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BODY TEMPERATURES OF IMMATURE WHITE LEGHORN COCKERELS INFECTED WITH NEWCASTLE DISEASE VIRUS*†

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IN STUDIES on the interrelation of diet and Newcastle disease virus (NDV) infection in immature White Leghorn cockerels (Squibb[1]), the possibility was explored that body temperatures might be used to predict the stage of active disease involvement. Body temperatures recorded at intervals during a 14-day period were compared with daily observations and related to the clinical syndrome.

PROCEDURES AND RESULTS

The birds in all three experiments were susceptible 4-week-old White Leghorn cockerels. An electric thermometer** with a needle thermocouple was used. Temperatures were taken at the same hour on each sampling day by inserting the needle into the chick's leg muscle; pilot studies indicated that this procedure gave representative and uniform results. Squibb[1] described the type ("H" strain NDV from Beaudette collection, Rutgers) and preparation of the virus, inoculation techniques, and confirmation of infection.

Experiment 1

For each of three replicated trials, 31–37 birds were inoculated with NDV, and body temperatures were taken at 0, 3, 7, 11, and 14 days, the times chosen to coincide with routine observations on growth and feed consumption. Data on the latter are reported in Squibb.[1] Body temperatures av-

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eraged 106.7°F before inoculation, 107.5° on the third day following inoculation, 105.4° on the 7th day, and normal again on the 14th day (Table 1). These small differences indicated that average measurements at the intervals designated were of little value.

Experiment 2

Individual birds in experiment 1 varied considerably in body temperature, and this variation might be related to the clinical syndrome. Therefore, 33 birds were infected with NDV, and body temperatures were again taken 0, 3, 7, 11, and 14 days after inoculation. The data were grouped as 1) recovered; 2) those with distinct involvement of the central nervous system (CNS), e.g., paralysis or torticollis; and 3) dead. As shown in Table 2, body temperatures of birds that recovered or still showed mild respiratory symptoms remained within normal ranges during the 14-day observation period; chicks with nervous involvement showed a slight rise in body temperature on the 3rd day, and then a return to normal levels. Where death resulted, however, a significant increase in body temperature was followed by subnormal values from which there was no recovery. This trial demonstrated that relating body temperatures to the NDV clinical syndrome was of value.

Experiment 3

Though the clinical syndrome showed a relation to body temperatures, intervals of 0, 3, 7, 11, and 14 days still did not

Table 1. Average body temperatures (°F) of NDV-infected immature W.L. cockerels.

Replicates	Days following inoculation				
	0	3	7	11	14
1	106.9(37)[1]	107.5(37)	104.7(28)	105.8(23)	106.5(20)
2	106.5(31)	107.4(31)	104.3(25)	105.9(22)	105.8(20)
3	106.7(32)	107.4(32)	106.5(22)	106.2(21)	105.8(21)
Av.	106.7	107.5	105.2	105.6	106.3

[1] Number of birds shown in parentheses

Table 2. Relation of average body temperatures (°F) to clinical symptoms in NDV-infected immature W.L. cockerels.

Symptoms at end of trial	No. birds	Days following inoculation				
		0	3	7	11	14
Recovered	12	107.1±0.13[1]	106.9±0.27	106.6±0.20	106.7±0.19	106.5±0.36
Nervous	13	106.8±0.12	107.1±0.14	105.8±0.51	105.7±0.43	105.6±0.19
Dead	8	106.9±0.19	108.1±0.29	101.1	—	—

[1] Standard error.

yield a difference great enough to predict the active stage of involvement of the disease. This experiment therefore was conducted to determine the value of taking body temperatures daily at the same hour. Two groups of cockerels were housed in separate, isolated quarters at an ambient temperature 68–72°F. One group (10 birds) was uninfected controls; the other group (32) was infected with NDV. Data from the birds were classified as follows: 1) uninfected controls; 2) immune, and recovered with no apparent symptoms; 3) immune, with respiratory symptoms; 4) immune, with respiratory symp-

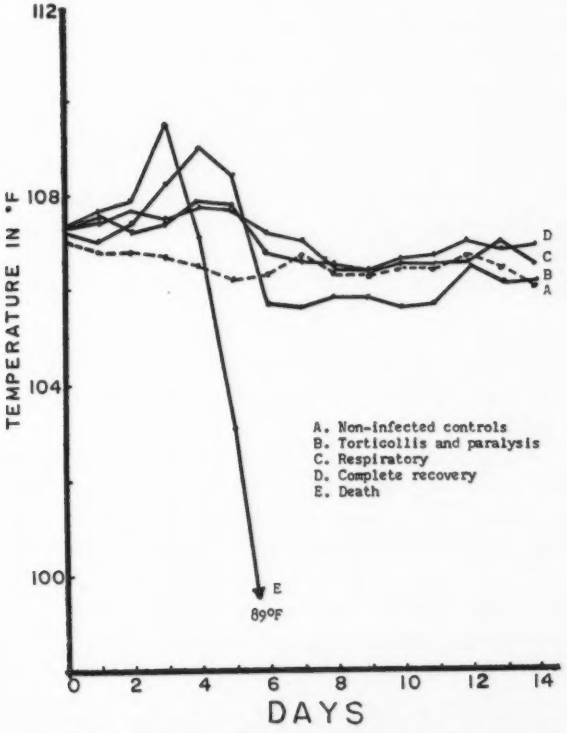

Fig. 1. Relation of clinical symptoms to body temperatures of 4-week-old White Leghorn cockerels infected with NDV.

toms and CNS involvement as indicated by paralysis and torticollis; and 5) dead.

Fig. 1. shows that daily body temperatures of the uninfected birds ranged from 107.0 to 106.0°F during the 14-day observation period. Birds that recovered from NDV infection or showed only mild respiratory symptoms had temperatures that did not fluctuate much beyond normal ranges. With CNS involvement, however, temperatures always rose significantly over more than 48 hours, and then dropped to abnormal levels.

SUMMARY

Body temperatures of immature White Leghorn cockerels infected with the "H" strain of Newcastle disease virus may serve to predict gross features of disease involvement if taken at a minimum of 24-hour intervals.

Temperatures that did not rise above 108°F were associated with birds that either recovered completely or showed only mild respiratory symptoms at the end of a 14-day disease infection period. Abnormally high temperatures (>108°F) lasting more than 48 hours, followed by a significant drop below normal levels (<106°F), were associated with acute neurotropic involvement, e.g., paralysis or death.

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STUDIES ON AVIAN ENCEPHALOMYELITIS. V. DEVELOPMENT AND APPLICATION OF AN ORAL VACCINE*

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AVIAN encephalomyelitis (AE) of chickens has been plaguing hatcherymen for a number of years. Two approaches toward reduction or elimination of losses from the disease have been reported. Schaaf and Lamoreaux[6] were able to demonstrate that AE could be controlled by vaccination, producing a lasting immunity similar to that following natural outbreaks. A second approach, suggested by Taylor and Schelling,[8] involved hatching only from flocks shown to have had the disease. The observation that many flocks become naturally infected with the virus during their lifetime makes such an approach feasible for hatcheries if they can afford to eliminate susceptible flocks as sources of hatching eggs.

Schaaf's work stimulated much activity toward the development of better and safer vaccines. Also of primary significance was the finding by Jungherr *et al.*[4] that AE virus could be adapted to produce lesions in chicken embryos. Those workers later reported[7] that only eggs from susceptible flocks could be used to demonstrate the presence of the virus. Thus, this research provided much of the knowledge and technique necessary for testing immunity to AE.

The development and use of embryo-susceptibility[7] and

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serum-neutralization (SN) tests[1] allowed the evaluation of various vaccines and vaccination procedures. It was found that certain isolates of AE virus, administered by wing-web or intramuscular inoculation, could immunize birds but sometimes produced paralysis.[2,5] Oral administration of the Van Roekel (VR) strain of AE could stimulate an immune response without the appearance of clinical signs.[2] However, the necessity of extremely high doses made this impractical.

Both the virus employed by Schaaf and the VR strain used in this laboratory had undergone many intracerebral passages in chicks. The VR strain, which was embryo-adapted, did not behave in birds as did viruses isolated from field outbreaks, i.e., it did not infect the intestinal tract, spread by contact, or produce clinical signs in susceptible day-old chicks following contact exposure.[2,3] On the other hand, epizootiological studies indicated that virus isolates that had undergone little laboratory manipulation and had not become embryo-adapted were capable of producing a syndrome indistinguishable from a natural outbreak.[3]

Avian encephalomyelitis is subclinical in young birds over 5 weeks old, but may be clinical in mature birds to the extent of a temporary drop in egg production.[9] An isolate (#1143) selected because of its ability to produce a "natural" type of infection in susceptible birds was employed in the trials reported here. The safety and efficacy of the virus as an oral vaccine were tested under both laboratory and field conditions.

MATERIALS AND METHODS

Isolate #1143 was obtained from the brains of clinically affected two-week-old chicks. It was propagated by serial passage in susceptible embryonating hen's eggs. Inoculation of 4-day-old embryos via the yolk sac was followed with harvest of embryo brain on the 19th day of incubation. After six such passages (1143-1 to 6) in embryos the virus was administered orally to susceptible day-old chicks. Brains were harvested from these chicks (1143-6+ Ch-1) upon the appearance of clinical signs (11–13 days postinoculation) and used to initiate three additional serial passages in embryos (1143 A-1 to A-3).

Three embryo-adapted virus isolates were also employed. These strains differed from #1143 virus to the extent that in addition to being capable of growth in susceptible eggs, they produced paralysis and muscular dystrophy in embryos similar to that described by Jungherr *et al.*[4] The VR strain has been described.[1] Isolate #86, obtained from a field case of AE in chicks, became adapted to produce lesions after eight yolk-sac passages. Isolate TAY (kindly supplied by Dr. J. R. E. Taylor, DeKalb Agricultural Association, Inc., DeKalb, Illinois) was embryo-adapted upon receipt. All virus was stored as 1:1 or 1:5 brain suspension at –20°C.

For titrations and virus assays of #1143 virus, one-to-five-day-old susceptible chicks were used. Each virus dilution or sample was inoculated into several chicks maintained in a modified Horsfall unit. Oral inoculations were done as follows: each tenfold dilution or feces suspension (1:1 in broth) was added at a rate of 1 ml per chick to 60 ml of distilled water. The mixture was then supplied to a group of chicks (usually 4 or 5) as drinking water. For intracerebral inoculations, a dose of 0.05 ml per chick was used. Criteria for infection in either case were ataxia by 17 days postinoculation, histological lesions at 17 or 18 days postinoculation, and in some instances positive serology at 28 days postinoculation. Since strain #1143 spreads easily from chick to chick within a unit, titration endpoints were necessarily determined as minimal infective doses (MID).

Titrations of the embryo-adapted isolates were done in susceptible embryos, and endpoints were determined as 50% embryo-infective doses (EID_{50}). Serological tests, embryo-susceptibility tests, and intracerebral-challenge techniques have been described.[1,2,3] For these tests, VR virus was employd.

Eggs for embryo passage, virus titrations and SN tests, and chicks used for laboratory trials were obtained from a privately owned flock of known susceptible White Leghorns.

EXPERIMENTAL

Characteristics of vaccine virus: No definite evidence of embryo-adaptation was observed either in the original six

embryo passages or in the three embryo passages done subsequent to a chick passage. In some passages, a slight to moderate degree of paralysis similar to that observed with the VR strain was noted in occasional embryos. These were not included in the brain harvests for routine passage or in the preparation of vaccine. An attempt to produce lesions typical of those described for VR virus by harvesting and passing from only paralyzed embryos failed in three successive passages.

Titrations in chicks were conducted on the fifth and all subsequent serial passages of #1143 virus. Results are in Table 1. It should be noted that virus titers were fairly consistent and that there was excellent correlation among clinical signs, histopathology, and serology. In most instances, it was found that 1 ml of stock virus contained approximately 1000 MID.

Table 1. Titrations of vaccine virus (#1143) by oral inoculation[a] of susceptible day-old chicks.

		11-16 da. P.I.	16-18 da. P.I.	4 wks. P.I.	Estimated
Virus passage	Virus dilution	Clinical signs	Positive histology	Positive serology	MID per ml
5	10^{-2}	4/4	2/2	2/2	
	10^{-3}	1/4	1/2	0/2	1,000
	10^{-4}	0/4	0/2	0/2	
6	10^{-2}	4/4	4/4		
	10^{-3}	2/4	3/4		1,000
	10^{-4}	0/3	0/3		
6+ Ch-1[b]	10^{-3}	3/3	3/3		1,000
	10^{-4}	0/4	0/4		
A-1[c]	10^{-1}	6/6	3/3	2/2	
	10^{-2}	5/6	2/3	3/3	100
	10^{-3}	0/6	0/3	0/3	
A-2	10^{-2}	5/5	5/5		1,000
	10^{-3}	5/5	5/5		
	10^{-4}	0/5	0/5		
A-3	10^{-2}	4/4	4/4		
	10^{-3}	3/4	4/4		10,000
	10^{-4}	1/4	2/4	-	

[a] Oral administration = 1 ml of virus dilution per chick in drinking water.

[b] Chick brain harvested following oral administration of 6th embryo passage.

[c] Embryo passages identified as A-1 to A-3 initiated from the chick passage, 6+ Ch-1.

Table 2. Titration of vaccine virus (#1143) and 2 strains of embryo-adapted virus (VR and TAY) by oral administration to 21-day-old susceptible chicks.

Virus	Dose[a] per bird	Serological response 14 da.	21 da.	28 da.	28 da. GMT	Lesions[c] 14 da.	21 da.	Virus in feces
1143 6th embryo passage	1 ml 10^{-2}	2/2[b]	2/2	2/2	2.1	1/2	2/2	+
	1 ml 10^{-3}	1/2	2/2	2/2	2.2	1/2	2/2	+
	1 ml 10^{-4}	0/2	1/2	2/2	2.2	2/2	1/2	+
	1 ml 10^{-5}	0/2	0/2	0/2	.3	0/2	0/2	—
VR	1 ml 10^{-0}	1/2	2/2	1/3	.6	0/2	0/2	—
TAY	1 ml 10^{-0}	0/2	0/2	1/3	.3	0/2	0/2	—
Control	None	0/2	0/2	0/3	.2	0/2	0/2	—

[a] 1 ml per bird pipetted directly into crop. VR virus titer in eggs, 1 ml = 1×10^{6} EID_{50}; TAY titer in eggs, 1 ml = 1.5×10^{6} EID_{50}.
[b] Fractions indicate number positive over number tested. NI of 1.1 or greater considered positive. GMT = geometric mean titer.
[c] Brain and gizzard examined histologically. Positive/examined.

Since titration by oral inoculation is not a conventional technique with AE virus, intracerebral and oral titers were compared with early-passage #1143 virus (1143-1). It was found that the oral titer was 10^{3} MID per ml whereas the intracerebral MID titer reached 2×10^{6} per ml. These results contrasted sharply with those observed with embryo-adapted virus. TAY virus (5th embryo passage) titrated to 2×10^{5} MID per ml by the intracerebral route, whereas undiluted virus failed to elicit a response by any criterion when administered orally. Results were similar with isolate #86 (12th embryo passage.)

Vaccine virus (1143-6) was titrated in 21-day-old susceptible chicks in another trial. The birds were held in modified Horsfall units, and tenfold serial dilutions were given per crop at a dose of 1 ml per bird. Six chicks were used for each dilution. For comparison, seven birds were each given 1.0×10^{6} EID_{50} of VR virus per crop, seven birds were given $1.5\times 10^{6} \times$ EID_{50} of TAY virus, and seven were uninoculated controls. All groups were tested serologically at 14, 21, and 28 days postinoculation. At 14 and 21 days postinoculation, two birds from each group were sacrificed for histological examination. Feces pools from each group collected from the 7th through the 10th days postinoculation were assayed for AE virus in susceptible chicks. Results are in Table 2. Although

responses to #1143 virus, both serologically and histologically, were good in all dilutions through 10^{-4}, response was only very sporadic and poor in groups given embryo-adapted virus (VR and TAY). Virus was demonstrated in the feces of all infected #1143 groups, but not in feces from the VR and TAY groups. No clinical signs were observed in any of the groups throughout the experiment.

Laboratory vaccination trials. Two laboratory trials were conducted to test the vaccine on a preliminary basis for safety, immunogenicity, and ability to spread by contact. Fifth-embryo-passage material was used as vaccine in both trials.

In the first experiment, 13-week-old susceptible chickens were used. To each of 20 birds, approximately 4 MID of virus #1143 was administered via the drinking water. These vaccinates were held in a sterilized battery for 48 hours postinoculation and then placed on the floor of an isolation unit with 19 susceptible contacts. Twelve uninoculated isolated controls were held in a separate unit. Serum-neutralization tests were conducted prevaccination and at 2, 3, 4, and 6 weeks postvaccination. The birds were observed for clinical signs throughout the experiment. One vaccinate was serologically positive at 2 weeks, whereas by 3 weeks postvaccination, over 50% of both the vaccinated and contact groups were immune. At 6 weeks postvaccination, 15 of 19 (79%) vaccinates and 13 of 17 (77%) contacts were positive by the SN test. Isolated controls remained negative throughout. No clinical signs were observed.

Nine-week-old susceptible birds were vaccinated in the second trial. Each of 10 birds received 1000 MID pipetted directly into the crop. They were immediately placed on the floor of an isolation unit with 18 contact birds. Eight birds maintained in another unit served as isolated controls and remained negative throughout the experiment. Again, over 50% of both vaccinates and contacts were serologically immune at 3 weeks. All were immune at 4 weeks postvaccination. As in the first trial, no birds developed clinical signs.

Vaccination—field trials. *Breeders.* Six flocks of mature birds involving approximately 5,000 birds were vaccinated

under field conditions. Age at vaccination ranged from 8 to 11 months. In each of 5 flocks the dose per bird was 10 MID; the proportion of birds vaccinated per flock varied from 1 to 100%. For the sixth flock, 100 MID was administered to 1% of the birds. When all birds in a flock were dosed, the vaccine was administered in the drinking water (an amount to be consumed in approximately 2 hours). Tap water without a stabilizer was used. When less than 100% of the birds were dosed, vaccine was administered by pipetting 1 ml of virus suspension (diluted in nutrient broth) directly into the crop. From one flock (A), eggs were collected daily and hatched weekly; the progeny were raised in isolation units to determine the incidence of egg and contact transmission. Eggs for embryo-susceptibility tests were collected from all flocks prevaccination and at various intervals postvaccination. Also from each flock (excepting flock F) chicks were hatched from eggs laid at least 4 weeks postvaccination. These, along with known susceptible controls, were challenged at one day of age via the drinking water with 100 MID per chick of #1143 virus. Resistance to this challenge was presumed to indicate parental immunity.

Table 3 gives data on the rate of egg transmission and contact transmission in flock A. On the basis of results reported,[3] chicks developing clinical signs during the first 7 days of age were considered infected via the egg. Those becoming ataxic after the 11th day of age were assumed to be infected from contact. It should be noted that only 1 of 58 chicks

Table 3. Incidence of clinical signs resulting from egg infection and contact infection in chicks hatched from a flock (A) of 11-month-old breeders vaccinated via the drinking water with #1143 virus.

Hatch	Days post-inoc. eggs collected	Number of chicks	Number developing clinical AE from egg transmission	Number developing clinical AE from contact exposure
1	1-7 da.	58	1[a] (1.7%)	34 (59%)
2	8-14 da.	64	3[b] (4.7%)	46 (72%)
3	15-19 da.	42	0	15 (36%)

[a] From egg laid 7 days postvaccination.
[b] Two from eggs laid 8 days postvaccination and one from egg laid 11 days postvaccination.

(1.7%) from eggs laid 1–7 days postinoculation and 3 of 64 (4.7%) from eggs laid 8–14 days postinoculation were infected by egg transmission. No egg-infected chicks appeared in the third hatch of 42 chicks (eggs from 15–19 days postinoculation). Those affected by contact exposure were respectively 59, 72, and 36% for the 3 hatches.

Table 4 contains data from all six flocks on numbers vaccinated, age, dose, and immune response as indicated by embryo-susceptibility and parental immunity tests. Tests with a minimum of 24 eggs from each flock indicated susceptibility prior to vaccination. Eggs resisting a challenge dose of 500–1000 EID_{50} VR virus were observed as early as two weeks postvaccination. By four weeks, the percentage of eggs resisting this challenge ranged from 65 to 93%, averaging 82%. It should be noted that the best response was observed in the flock (F) where each of 1% of the birds was dosed with 100 MID *per os*. The percentage of chicks demonstrating parental immunity varied among 5 flocks from 67 to 100% (average 84%).

No clinical signs following the vaccination of these 6 flocks were noted except for a drop in egg production in three instances (flocks A, C, and F). Flock A had been steadily decreasing in production prior to vaccination. A sharp decline of about 7% was noted on the eighth day postvaccination, and the rate of production continued to decrease as observed before vaccination. Flock C started decreasing in production on the 8th day postinoculation, dropped a total of approximately 12% (of 100% production) by the 10th–12th days, and returned to a normal rate by the 18th–20th day postinoculation. Egg production in flock F was lowered between the 11th and 18th days postinoculation. In this instance the maximum depression in production was about 20%. No significant changes in the rate of egg production following vaccination were observed in in flocks B, D, and E.

Growing birds. Fourteen flocks comprising over 63,000 birds were vaccinated prior to egg production. One flock was vaccinated at 16 weeks old, one at 19 weeks, and twelve between 5 and 7 weeks. Serum-neutralization tests indicated all flocks to be susceptible at vaccination. Vaccination in all

Table 4. Results of oral vaccination of six susceptible flocks of mature birds with AE virus #1143.

Flock	No. birds in flock	Age (mo.)	No. vacc.[a] (%)	Method of admin.	Percent immune eggs[b] 2 wk	4 wk	8-12 wk	Oral challenge, day-old chicks[c] Eggs collected for hatching	No. immune / No. chall.	% immune
A	500	11	500 (100)	water	40	84	95	4 wk postinoc.	54/54	100
B	550	9½	550 (100)	water	26	83	79	11 wk postinoc.	17/21	81
C	1,600	8	1,600 (100)	water	41	67	—	6 wk postinoc.	17/21	81
D	440	8	48 (11)	*per os*	25	65	78	5 wk postinoc.	12/17	71
E	475	9	5 (1)	*per os*	6	89	78	11 wk postinoc.	12/14	86
F	2,000	10	20 (1)	*per os*	—	93	—	Not done		

[a] Vaccine = Flock A - #1143, 5th embryo passage, 10 MID per bird.
Flocks B, C, D, E, - #1143, 6th embryo passage, 10 MID per bird vaccinated.
Flock F - #1143 passage A-3, 100 MID per bird vaccinated.

[b] Eggs challenged via the yolk sac with 500 to 1000 EID_{50} embryo-adapted (VR) virus.

[c] Control chicks hatched and challenged along with test chicks. 100 MID #1143, 6th embryo passage virus/chick administration via drinking water. Known susceptible controls succumbed to the extent of 94 to 100% in the five groups.

flocks consisted of *per os* administration of virus to 2% or less of the birds. Dose per bird varied from 25 to 250 MID among the various flocks. Blood samples for SN tests were collected from the 16-week-old flock at 4½ weeks after vaccination. With the other flocks, serological tests were done on both vaccinates and contacts at 3 weeks, and only on contacts at 6 or 7 weeks postvaccination. A third postvaccination test was done on the 12 younger flocks at approximately 3½ months. Intracerebral challenge of 10 birds from each of the 12 younger flocks was done at vaccination and again at 7 weeks postvaccination. The challenge dose was 10,000 EID_{50} VR virus per bird. These birds were selected at random from those bled for SN tests.

Table 5 gives data from the trials on the 5- to 7-week-old flocks. Of samples tested from these 12 flocks, 32 of 112 (29%) of the vaccinates and 5 of 120 (4%) of the contacts were immune serologically at 3 weeks postvaccination. At 7 weeks, 124 of 180 (69%) were immune serologically. Intracerebral challenge, also done at 7 weeks, was resisted by 110 of 119 (93%) of the birds. It should be noted that only birds with an N.I. of 1.1 or greater were considered immune, and that, as previously observed,[2] birds considered negative serologically may resist challenge. On the other hand, no bird with an N.I. of 1.1 or greater succumbed to challenge. Results from SN tests at 3½ months postvaccination indicated that 102 of 120 (85%) were serologically positive.

The 19-week-old flock responded immunogenically much more rapidly than did the younger flocks. By 21 days postinoculation, all 8 vaccinates tested and 9 of 10 contacts were positive by the SN test. At 6 weeks, 14 of 15 (93%) of the contacts were immune with a GMT of 2.1. Serological tests done on the 16-week-old flock indicated that 17 of 20 (85%) were immune (GMT 1.8) at 4½ weeks postvaccination. In these flocks, as well as in the 12 younger flocks, no clinical signs attributable to AE were observed after vaccination.

In view of the difference in rapidity of response between the younger (5–7 weeks) and older (16 and 19 weeks) groups, a trial was conducted to determine the effects of age and parental immunity on response to oral vaccination. Eggs were collected from a privately owned flock of White Rocks known to

Table 5. Results of oral vaccination with AE #1143 virus of 12 flocks of susceptible growing birds.

Flock	Age	No. birds	No. vacc.	Dose[a] (MID)	Serology—positive/tested (GMT)					Intracerebral challenge	
					Prevacc.	3 wk postvaccination		7 wk P.V. contact	3½ mo P.V. contact	Prevacc.	7 wk P.V.
						Vacc.	Contact				
1	34 da.	3,500	20	200	0/10(0)	N.D.	N.D.	12/15(1.7)	8/10(1.5)	10/10	0/10
2	41 da.	5,000	20	200	0/10(.3)	3/8(.6)	0/10(.1)	11/15(1.1)	7/10(1.4)	10/10	0/9
3	41 da.	2,500	20	200	0/10(.3)	2/9(.3)	0/10(.1)	5/15(.7)	8/10(1.7)	8/8	2/10
4	42 da.	5,000	20	250	0/10(.2)	2/20(.5)[b]	0/10(.4)	8/15(1.1)	9/10(1.6)	10/10	3/10
5	43 da.	3,000	20	250	0/10(.2)		0/10(.2)	6/15(.7)	8/10(1.7)	10/10	2/10
6	45 da.	2,500	20	200	0/10(.2)	3/8(.6)	0/10(.1)	13/15(1.3)	9/10(1.6)	10/10	0/10
7	45 da.	5,000	20	200	0/10(.1)	N.D.	N.D.	12/15(1.4)	8/10(1.2)	9/9	1/10
8	45 da.	4,000	20	25	0/10(.3)	4/9(1.0)	1/10(.5)	11/15(1.3)	8/10(1.3)	10/10	0/10
9	47 da.	6,500	20	200	0/10(.3)	4/9(.6)	0/10(.1)	14/15(1.6)	9/10(1.5)	10/10	0/10
10	48 da.	6,000	20	200	0/10(.4)	1/10(.3)	0/10(.1)	12/15(1.2)	8/10(1.5)	10/10	0/10
11	49 da.	5,000	20	250	0/10(.2)	10/10(1.9)	4/10(1.0)	11/15(1.5)	8/10(1.6)	10/10	0/10
12	50 da.	6,000	20	250	0/10(.1)	2/10(.6)	0/10(.4)	9/15(1.1)	10/10(1.5)	10/10	0/10

[a] Flocks 4, 5, 11, and 12 vaccinated with #1143 A-2 embryo passage virus; flock 8 with #1143 A-1, all others with #1143 6th embryo passage virus. Vaccine administered *per os*.

[b] These samples mixed per error.

have undergone a recent outbreak of AE. Of 22 eggs tested, all resisted challenge with 1,000 EID_{50} VR virus. Eggs from the immune dams were incubated and hatched in isolation with eggs from a known susceptible flock of White Leghorns. All chicks were raised together in an isolation unit. Birds from each group were removed at intervals for SN tests and oral challenge with 100 MID of #1143-6 virus. Post-inoculation SN tests were done at 21 days after challenge to compare responses. Feces were collected from each group between the 7th and 10th day postinoculation and assayed for AE virus in susceptible chicks. The results are in Table 6. Parental antibodies were detected by the SN test in the chicks from immune dams at 4 weeks but not at 6 weeks of age. It was not until the 10th week of age, however, that vaccination of chicks from immune dams induced serological responses comparable to those observed in chicks from the susceptible source. Clinical signs following oral challenge were noted only in the susceptible group inoculated at 4 days old. Attempts to demonstrate AE virus in the feces following oral challenge were successful in each of the first three lots (4 days, 4 weeks, and 6 weeks old) of susceptible source chicks. Chicks from the immune source

Table 6. The influence of age and parental immunity on serological response and virus excretion following oral inoculation with vaccine virus (#1143).

Group[a]	Age	SN tests[b]		Virus isolation from feces[c]
		Prevacc.	3 wk postinoc.	
I A	4 da.	10/10(1.4)	0/6(.4)	Negative
I B	4 da.	0/10(0)	6/6(1.6)	Positive
II A	4 wk	3/10(.4)	0/10(.3)	Positive
II B	4 wk	0/10(.1)	10/10(1.6)	Positive
III A	6 wk	0/10(.2)	0/7(.2)	Positive
III B	6 wk	0/10(.1)	7/7(1.4)	Positive
IV A	8 wk	0/10(.1)	3/10(.8)	Not Done
IV B	8 wk	0/10(0)	7/(2.0)	Not Done
V A	10 wk	0/9(0)	9/9(1.8)	Not Done
V B	10 wk	0/9(.1)	9/9(1.9)	Not Done

[a] A = chicks from immune dams; B = chicks from susceptible dams.
[b] Fractions = number positive over number tested. Numbers in parentheses = geometric mean titer.
[c] Feces pools collected 7 to 10 days postinoculation.

vaccinated at 4 days old did not subsequently excrete detectable virus, but virus was detected in the feces of the groups inoculated at 4 and 6 weeks old.

DISCUSSION

Of all the major virus diseases of poultry, avian encephalomyelitis is, in growing birds, perhaps the most innocuous. The subclinical nature of the infection in birds over 3 or 4 weeks old and before they come into egg production, makes feasible immunization by natural type of infection. This paper presents data on the use of a vaccine that, as far as could be distinguished, produces just such an infection.

The virus used in preparation of the vaccine was one that had undergone comparatively few embryo passages. The necessity of low passage, or at least nonembryo-adapted virus, was illustrated in the trials using embryo-adapted strains. It would appear that, upon adaptation, AE virus not only gains the ability to produce characteristic lesions in susceptible embryos but also loses the ability to infect via the intestinal tract. In an effort to avoid adaptation, the vaccine virus was passaged by oral inoculation of chicks after the sixth embryo passage. Assuming that embryo adaption follows a process of mutation and/or selection, it was further assumed that harvest of brain from orally inoculated chicks would screen out undesirable adapted virus. The vaccine did not lose the ability to infect via the intestinal tract and spread from bird to bird after the initial six serial passages in embryos or after the three embryo passages subsequent to the chick passage.

In evaluation of vaccine, safety is paramount. The virus used in these studies (isolate#1143) was given orally to over 63,000 birds between 5 and 19 weeks old, and to 5,000 breeders. In the growing birds, no postvaccination reactions were observed. Three of the six breeder flocks immunized decreased temporarily in egg production, this being the only adverse effect noted. Egg transmission occurred following vaccination. Both the egg transmission and the production drops followed patterns similar to those observed in both natural and experimental infections.

The value of a vaccine is naturally dependent upon its

immunogenic properties. Four different criteria were used for evaluating immune responses in the trials reported. In younger birds, SN tests and resistance to intracerebral challenge were employed. Determinations of immune responses in breeders were done with the aid of embryo-susceptibility tests and tests for parental immunity of progeny. It is conceded that results from these methods cannot be strictly compared to the degree of immunity needed against natural infection. However, it was assumed that each of the means employed for testing immunity was a more severe test than natural exposure and would underrate rather than overrate the degree of protection. Serum-neutralization tests used in 12 flocks vaccinated at 5–7 weeks old indicated approximately 69% to be immune at 7 weeks postvaccination. Intracerebral challenge on the same birds at the same time was resisted by 93%. By 3½ months after vaccination, 85% of the birds tested from the same flocks were immune to the SN test. These results are not dissimilar from those observed following many natural outbreaks, although they are not as striking as those obtained from flocks of 16- and 19-week-old birds. In those flocks, 85 and 90% of the samples tested were immune at 4½ and 3 weeks postvaccination, respectively. In view of the results obtained in the trial on parental immunity, where it was observed that birds from immune dams were not fully susceptible to oral inoculation until the 8th–10th week of age, it would appear that the 12 younger flocks (5–7 weeks old) were vaccinated at an earlier than optimal time. Although the responses apparently were not prohibited, they were delayed for a period.

Embryo susceptibility tests following vaccination of breeders indicated that virus spread was rapid, immune eggs being detected as early as 2 weeks postvaccination. There was little difference in the average percentage of immune eggs at 4 weeks as compared to 8–12 weeks postvaccination (about 82% for each test period). Tests for parental immunity of progeny from eggs laid 4–11 weeks postvaccination were positive with approximately 84% of the chicks tested.

Few variables were used in the trials reported: doses varied from 4 to 1000 minimal infective doses (MID) per

bird; the percentage of birds vaccinated within a flock ranged from 0.3 to 100%; administration was either drinking water or *per os* inoculation (pipetted directly into the crop). In the 2 laboratory trials, the response following administration of 1,000 MID exceeded that following vaccination with 4 MID. In the latter group, 75% of the birds developed immune SN titers (GMT of 1.7); in the former 100% had immune titers (GMT of 2.4). The results were essentially the same in both contacts and vaccinates. In breeder flocks (field trials), 10 MID per bird initiated infections whether 1, 10, or 100% of the birds were dosed. Results at 4 weeks postvaccination were comparable regardless of the number vaccinated per flock. The route of administration (water or *per os*) was not found to be instrumental in determining the degree of serological response. It would appear that, once an infection is established within a susceptible flock, it runs its course as does the natural infection.

A third consideration to be made is one of practical significance—that is, economic feasibility. The vaccine must be propagated and titrated in fully susceptible embryos. However, once the susceptible status of an egg supply is established, production is no more difficult or expensive than for most other vaccines. An average of 0.75 ml of brain material was obtained from each embryo harvested. After a dilution of 1:1, the virus suspensions were found to contain roughly 1,000 MID per ml. Assuming that an optimum per-bird dose is of the order of 100 MID (allowing for a margin of safety) and that it is necessary to vaccinate only 1% of the birds in a flock, the virus yield per embryo is adequate for vaccination of a flock of 1,500 birds.

ACKNOWLEDGMENT

Appreciation is expressed to Dr. George P. Faddoul for supplying the field case material from which this work was initiated.

SUMMARY

The safety and efficacy of a live-virus oral vaccine for avian encephalomyelitis were tested. Results from two laboratory trials and twenty field trials are reported. In all, over

63,000 growing birds and 5,000 breeders were vaccinated. No clinical signs were noted subsequent to vaccination except for a drop in production in 3 of 6 breeder flocks inoculated. Vaccine was administered in some instances in the drinking water, in others by *per os* dosage of a few (1% or less) birds within the flock. In the latter instances, virus dissemination in a given flock was rapid and thorough. Immune responses following either method were comparable to those observed following natural outbreaks of AE. Because of the possible interference of parental immunity in birds under 10 weeks old, and egg transmission in breeders, it is recommended that vaccination be done between the tenth week of age and 3 weeks prior to egg production.

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TISSUE CULTURE PROPAGATION AND PATHOLOGY OF CELO VIRUS*

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THE first reported isolation of a chicken embryo lethal orphan (CELO) virus was made by Yates and Fry,[3] in 1952. They made several isolations from chicken embryos while attempting the isolation of other agents. In addition Dubose and Grumbles[2] demonstrated that the CELO virus could cause quail bronchitis. This communication reports the propagation and pathology of a CELO agent in cell cultures and chicken embryo tissues; this agent was isolated in chicken-embryo kidney-cell cultures during serial passages of infectious laryngotracheitis (ILT) virus. The commercial ILT vaccine virus had been propagated for 16 serial passages in chicken-embryo kidney-cell cultures and caused characteristic cytopathic effect (CPE).[1] The 17th passage produced a totally different type of CPE. Since this CPE strongly suggested a change in the nature of the virus, further studies were performed.

MATERIALS AND METHODS

The chicken-embryo kidney-cell cultures were prepared and the plaque techniques and virus neutralization tests conducted as previously reported.[1]

UConn CELO virus. The virus hereinafter referred to as UConn CELO virus was obtained from the 17th cell culture passage of a commercial ILT virus vaccine. This passage was referred to as the 1st cell culture passage. The 3rd cell culture passage was plaqued in monolayer cell sheets, and a single plaque was picked for the 4th cell culture passage. The 5th cell

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culture passage produced mortality when inoculated into the chorioallantoic sac (CAS) of 9-day-old embryonating chicken eggs.

This virus, grown in embryos or cell culture, was not neutralized by specific antisera for ILT, Newcastle disease (ND), infectious bronchitis (IB), or avian encephalomyelitis (AE) viruses. Antiserum prepared for this agent neutralized the Phelps CELO agent (The Phelps CELO agent[3] was obtained from Dr. V. J. Yates, University of Rhode Island, Kingston, Rhode Island) but did not neutralize ILT, ND, IB, or AE viruses. Preimmunization serum was negative to all these viruses, including the Phelps CELO virus. These serologic and cultural characteristics, along with the findings presented in this paper, led the authors to the name UConn CELO virus.

Virus propagation. The 5th cell culture passage of the UConn CELO virus was used to inoculate the CAS of 9-day-old embryonating chicken eggs. The agent was subsequently serially passed 12 times in the CAS of embryonating eggs by inoculating 0.1 ml of infected allantoic fluid diluted 10^{-3} in tryptose broth. This agent was also serially passed in chicken-embryo kidney-cell cultures.

Serum production. Eight-week-old White Rock (WR) birds were bled for preinoculation serum and inoculated in the vent by the brush method with undiluted 8th cell-culture-passage UConn CELO virus. Three weeks later, the birds were challenged with the same material by inoculating 0.2 ml into the infraorbital sinus (i.s.). The birds were bled for serum 1 week later.

A second trial was made with 10-week-old WR birds using the same inoculum and schedule. In this trial, noncontact control birds were kept in the same room. All birds were bled prior to inoculation and at 4 weeks postinoculation.

CAM sections. Nine-day-old chicken embryos were inoculated with 0.1 ml of undiluted 8th cell culture passage UConn CELO virus. Each day for 7 days postinoculation, CAM sections from 3 embryos were fixed in acidified Zenker's, dehydrated, embedded in paraffin, cut at 6 μ, and stained by hematoxylin and eosin. This work was duplicated using the 2nd cell culture passage Phelps agent.

Permanent slides of cell cultures. Cultures grown on cover slips contained in Leighton tubes were infected with

either 8th UConn of 2nd Phelps cell culture passage CELO viruses. When about half of the cells in the culture showed CPE the cells were fixed in Bouin's or acidified Zenker's fixative for 1 hour, washed in tap water, and stained by hematoxylin and eosin. The cover slips were then mounted permanently on glass slides. To demonstrate intranuclear inclusion bodies, other cultures were stained when all cells appeared to be infected.

RESULTS

Pathologic alterations. Both the UConn and Phelps CELO viruses produced CPE in chicken-embryo kidney-cell cultures in about 18 hours. In tissue-culture systems the CELO virus affected individual cells, causing the nucleus to enlarge and become filled with fine basophilic granules (Fig. 7). The effect culminated at 30–40 hours postinoculation with the formation of intranuclear inclusion bodies of compact granules. In contrast, the ILT virus caused pale acidophilic intranuclear inclusion bodies, which were always more sharply defined. Furthermore, these inclusions were less common in the material examined. In addition the ILT virus caused a characteristic clumping of the cells suggesting an agglutination phenomenon (Fig. 8). In contradistinction to fixed paraffin-blocked tissue, Zenker's fixative was a poor second to Bouin's fixative in bringing out architectural characteristics.

UConn CELO and Phelps cell-culture-passed viruses produced visible lesions when inoculated on the CAM of 9-day-old chicken embryos. The gross lesions first appeared in 2 days, progressed along the blood vessels, and often became confluent by 6 days postinoculation. These lesions were very similar in gross appearance to those caused by ILT virus.

The first visible histologic lesion appeared in 2 days and consisted of diffuse ectodermal hyperplasia with concomitant mesodermal reaction of mesenchymal elements. This is an embryonic expression of phagocytosis and inflammation. Striking were prominent basophilic granular intranuclear inclusion bodies. From this point on, the progress of the lesion was in the direction of degeneration, resulting in ectodermal necrosis with hemorrhage and an increase of the phagocytic activity of the mesodermal cells (Figs. 2, 3, 4, 9).

The ILT affected the CAM in essentially the same manner, but a tendency toward localization of the affected area was noted in the ectodermal lesion. Above all, acidophilic intranuclear inclusion bodies frequently occurred in this condition providing an unmistakable pathognomonic signpost (Figs. 5, 8, 10). Differentiation between the two virus alterations became impossible after the third and fourth day owing to the tendency of both to cause necrosis.

Virologic studies. The 8th cell-culture passage of UConn CELO and the 2nd cell-culture passage of Phelps CELO caused plaques in tissue-culture cell sheets, and respectively produced titers of 10^{-9} and 10^{-8} plaque-forming units (PFU) per ml. Plaques could be counted 5 days postinoculation, at which time they were 0.5 to 1.0 mm in diameter; by the 12th day there were 6.0 mm, and at 28 days had become 10.0 to 11.0 mm. Plaques produced by these CELO viruses had a continuous regular edge (Fig. 11). In contrast, ILT virus plaques have an irregular edge. UConn CELO antiserum neutralized 10^6 PFU of homologous virus and 10^5 PFU of the Phelps virus.

The 5th cell-culture passage of UConn CELO was lethal for 9-day-old chicken embryos via CAS inoculation, and by the 3rd passage titered 10^{-10} ELD_{50} when 0.2 ml was inoculated per embryo. Comparable passages of Phelps CELO titered at 10^{-9} ELD_{50}. UConn CELO antiserum neutralized at least $10^{6.3}$ of either virus.

Birds inoculated with UConn CELO virus by the vent

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Figs. 1–8. 1) Normal 9-day-old chicken embryo chorioallantoic membrane (CAM), low power. *2.* CAM infected with CELO virus, 2 days postinoculation (pi), low power. Note CAM is infiltrated, and on the ectodermal surface is a localized focus of hyperplastic epithelium. *3)* CAM infected with CELO virus, 5 days pi, low power. In this view the ectodermal reaction has covered a much larger area. *4)* CAM infected with CELO virus, 10 days pi, low power. The ectoderm is almost completely lost owing to necrosis. Note marked mesodermal reaction. *5)* CAM infected with ILT virus, 3 days pi, low power. Although hyperplasia of ectodermal elements is still discernible, the necrotic propensities of the virus are already apparent. *6)* Normal chicken-embryo kidney cells (CEKC), low power. *7)* ×600 view of a CEKC, 2 days pi, infected with CELO virus. Large areas of the cytopathic effect are clearly visible; the large dark areas represent the basophilic granular accumulations, which actually are intranuclear. *8)* ×600 view of a CEKC, 2 days pi, infected with ILT virus. Large aggregates of cells simulating the agglutination effect, which is typical of ILT virus.

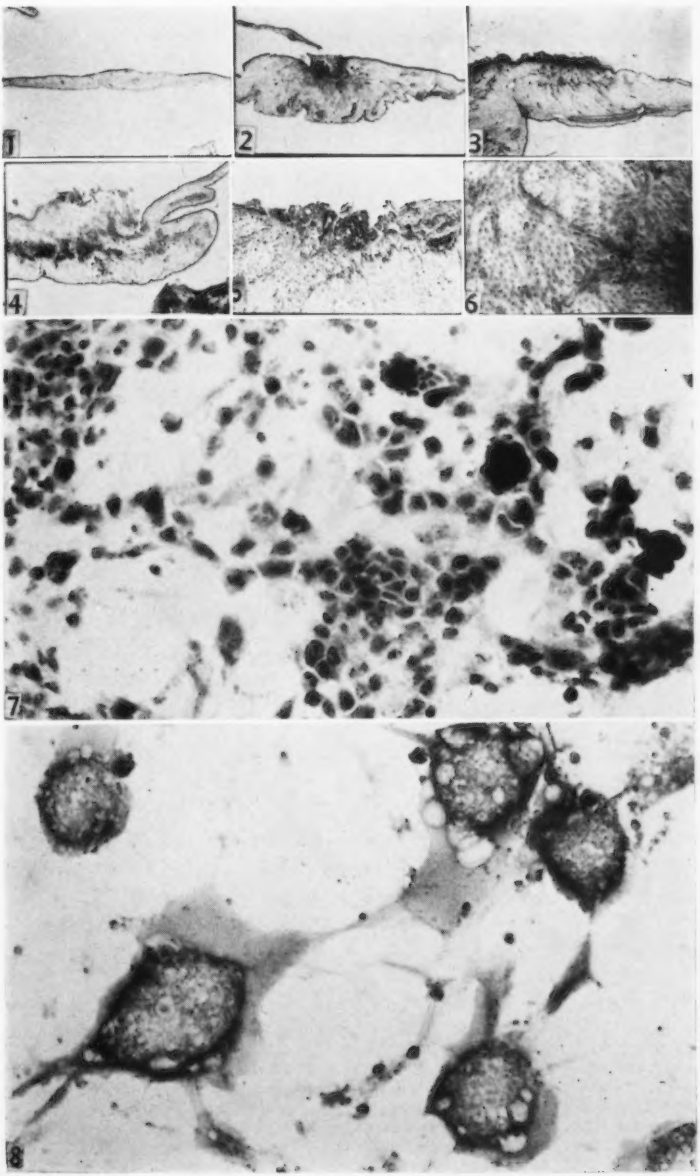
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Table 1. Comparative neutralization indexes of serums tested in chicken embryos and cell cultures

Immunization trial	Virus	Pre-inoc.	Post-inoc.	Control*
1	UConn (CAS)	$10^{0.5}$	$10^{7.5}$	—
1	UConn (CC‡)	10^{0}	$10^{6.0}$	—
1	Phelps (CAS)	$10^{0.8}$	$10^{6.3}$	—
1	Phelps (CC)	10^{0}	$10^{5.0}$	—
2	UConn (CAS)	$10^{0.2}$	$10^{7.0}$	10^{2}
2	Phelps (CAS)	$10^{0.4}$	$10^{7.2}$	10^{3}

* Taken at time postinoculation serum taken.
—Not done.
‡ Cell culture

brush method and challenged via the intraorbital sinus produced antiserum to both UConn and Phelps CELO viruses. The neutralization indexes (NI) of the pre- and postinoculation serums were respectively $10^{0.5}$ and $10^{7.5}$ to the UConn virus, and $10^{0.8}$ and $10^{6.3}$ to the Phelps virus (Table 1). In the second trial, the NI's of the preinoculation, the postinoculation, and the control serums were respectively $10^{0.2}$, $10^{7.0}$, and $10^{2.0}$ to the UConn virus, and $10^{0.4}$, $10^{7.2}$, and $10^{3.0}$ to the Phelps virus. The control serum was taken at the time the postinoculation serum was taken.

No gross lesions were noted in the vents of susceptible birds after infection with UConn CELO virus by the vent brush method. No clinical signs appeared in birds infected with this virus during immunization.

DISCUSSION

The source of the UConn CELO virus was not determined. One possibility is that it existed in the original ILT vaccine. This appears unlikely, however, since the CELO virus is known to grow more rapidly, and to a higher titer, in chicken-embryo kidney cells than does the virus of ILT.

Another possibility is that the virus was endogenous to the embryonic tissue. It would appear that this CELO virus

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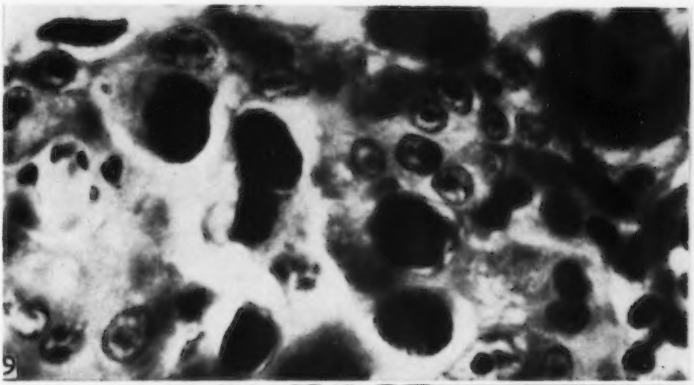
Figs. 9–11. 9) Oil immersion view of a CELO infected CAM, 2 days postinoculation. Only the ectoderm is shown. Though many normal cells are seen, others are overcome by the large basophilic intranuclear inclusions. *10)* ×400. High-power view of an ILT-infected CAM, 3 days postinoculation. This view shows both ectoderm and a small portion of the mesoderm. The pathognomonic intranuclear inclusion bodies of ILT, which are acidophilic, are quite numerous. *11)* Titration of CELO virus in CEKC culture by the plaque method.



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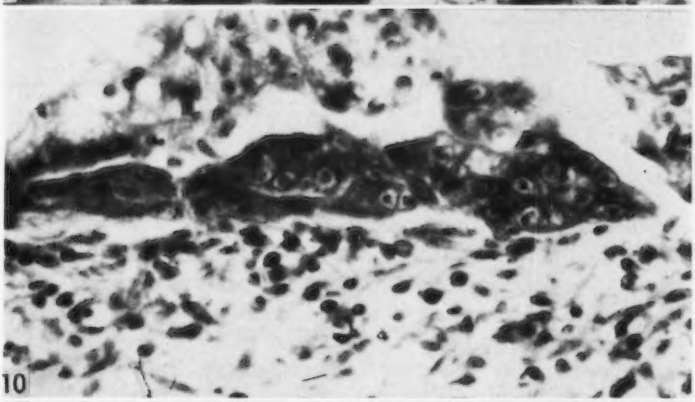

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was latent and did not cause CPE until the medium was changed and the culture stressed with inoculation of ILT virus. The CELO status of the hens producing the embryos used for cell culture was not known, since the flock was disposed of before the virus was identified.

Serologically and histopathologically the UConn isolate is similar to the Phelps virus. The latter, however, titers one log lower than the UConn agent in CAS and cell cultures. The Phelps virus is the more lethal of the two for embryos inoculated CAM.

Plaque formation by CELO virus in cell culture makes available a method for selecting a pure strain by picking and propagating from one discrete plaque. Since plaque formation is inhibited by specific serum, a VN test can be performed.

CELO virus infection in chickens is apparently of a nonclinical nature when the virus is introduced via the vent. Even with the spread of the virus to control birds, as occurred in the second immunization trial, no clinical signs were present.

SUMMARY

A CELO agent was isolated in a chicken-embryo kidney-cell culture during serial passage of infectious laryngotracheitis vaccine. The cytopathic effect of the CELO agent affected individual cells, causing the nucleus to enlarge and become stippled. Plaques were formed in cell sheets by the virus. Visible lesions were produced by the CELO virus on the chorioallantoic membrane of chicken embryos. Histopathologic examination revealed large basophilic intranuclear bodies 48 hours postinoculation. Virus neutralization tests were performed by the cell-culture plaque method and chicken-embryo allantoic-sac inoculation. Neutralization indexes were demonstrated of at least 10^5 plaque-forming units for the plaque method, and at least $10^{6.3}$ ELD_{50} for the CAS method.

The UConn CELO agent was identified by serologic and histopathologic comparison to a known CELO (Phelps) agent.

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EASTERN ENCEPHALITIS VIRUS IN DUCK EMBRYO FIBROBLAST CULTURE

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EASTERN encephalitis virus (EEV) has been propagated and has produced cytopathogenic changes in cultured fibroblasts of chickens embryos,[1,10] rats,[5,2] and mouse embryos.[11] It has been stated[7] that Pekin duck embryo cell cultures supported plaque formation by certain group A arbor viruses but that cytopathogenicity of the viruses in fluid cultures was questionable or undetectable. No other information accompanied that statement. An attempt was made to propagate the virus of EE from ducklings[3] in duck embryo fibroblast cultures. The results are reported here.

MATERIALS AND METHODS

Trypsin-dispersed[13] cells of mixed tissue origin from decapitated ten-day-old duck embryos were seeded 6×10^5/tube in 1 ml of growth medium,[12] and incubated. The outgrowth of continuous fibroblastic sheets, which developed within 24 hours of incubation, were inoculated with the EE virus of duckling origin that had prevously been carried ten passages in duckling kidney cell cultures.[9] Ten serial passages of the virus in the fibroblast cultures, titration of the virus in chicken embryos and in duck embryo fibroblast cultures and the identification of the fibroblast culture 10th passage virus by *in vitro* virus neutralization tests with normal and EE-immune sera (received from Dr. D. Stamm, CDC, Montgomery, Alabama) were performed following the same technique as was previously described for the passage of the virus in duckling kidney cell cultures.[9]

Plaque assay of the fibroblast-culture 10th-passage virus was performed in bottle cultures,[8] but the overlay medium

was in Hanks' salt solution[6] with 0.07% sodium bicarbonate, and contained 10% calf serum. Plaques were measured and counted daily. Plaque neutralization tests were carried out with 1:1 diluted normal or EE-immune horse serum (also recieved from Dr. Stamm).

In one experiment, which was designed to show if there was any correlation between the intensity of cytopathogenic changes and the magnitude of virus propagation, each culture was inoculated with 10^4 fibroblast culture infective doses of the duck embryo fibroblast culture 10th-passage virus. Control and virus-inoculated cultures grown on coverslips in Leighton tubes were harvested every four hours post-inoculation. At least four cultures of each were harvested each time. Harvested cultures were washed once with phosphate-buffered saline,[4] fixed in absolute methanol, and stained with hematoxylin and eosin. The fluid of virus-inoculated cultures from each harvest was pooled and titrated in duck embryo fibroblast cultures.

RESULTS

Cytopathogenicity of the EE virus for duck embryo fibroblasts was observed in all ten passages. During the first three passages, the majority of the nuclei in the virus-inoculated cultures became highly refractive after 24 hours of incubation. Near the end of 48 hours' incubation, cytoplasmolysis was extensive, leaving numerous naked nuclei with some cytoplasmic threads and spikes attached. There were numerous open spaces and large quantities of cellular debris floating in the medium. On these three passages, harvests were made at the end of 48 hours' incubation. The cytopathogenic changes were intensified through passage in the cell cultures. Cytoplasmolysis was extensive within 24 hours of incubation (Fig. 1) and from the 4th to the 10th passages harvests were made after less than 24 hours of incubation, at which time there were only naked nuclei on the glass. The seed virus, which was the 10th passage of duckling kidney cell-culture, had a titer of $10^{-5.0}$/0.2 ml in chicken embryos, and $10^{-1.7}$/0.1 ml in the duck embryo fibroblast cultures; and the respective titers were $10^{-4.0}$/0.2 ml and $10^{-5.0}$/0.1 ml after ten serial passages

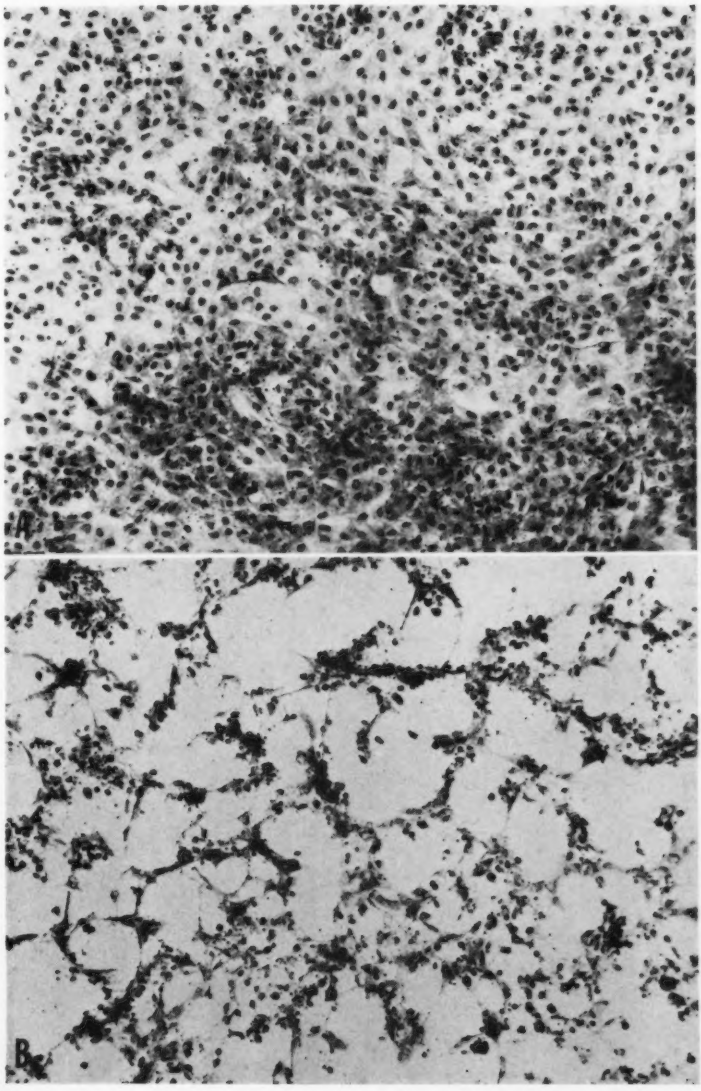

Fig. 1. A) Control culture of duck embryo fibroblasts, after 20 hours' incubation in maintenance medium. ×100, H&E stain. *B)* Duck embryo fibroblast culture inoculated with the 9th-passage virus, 20 hours post-inoculation. ×100; H&E stain.

in duck embryo fibroblast cultures. The cytopathogenicity of the passage virus was completely neutralized by EE-immune serum but not by normal serum.

Plaques about 1 mm in diameter became recognizable on the 2nd day of incubation. They gradually grew to 2–4 mm, with the majority about 3 mm in diameter, on the 4th day of incubation. They were nearly circular, with serrated margins (Fig. 2). The number of plaques in the cultures increased to the 3rd day of incubation; there was no significant increase in plaque count thereafter (Table 1). The plaque-forming end point of the virus was $10^{-5.0}$/0.1 ml, and it was estimated that 1 ml of the virus inoculum contained 4.3×10^6 plaque-forming units. Cultures inoculated with undiluted virus and 1:1 diluted EE-immune serum mixture had no plaque after three days of incubation, whereas those inoculated with the mixture of normal serum and 10^{-5} virus dilution had 2.8 plaques per culture. An average of four cultures was observed. The four

Fig. 2. EEV plaques in duck embryo fibroblast culture, 3 days post-inoculation. From left to right, control culture and cultures inoculated with 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} virus dilutions.

Table 1. Number of plaques formed by fibroblast-culture 10th-passage virus.

Days of incubation		Number of plaques produced by virus dilution[a]			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	Expt. 1	0	0	0	0
	2	0	0	0	0
	Av.	0	0	0	0
2	1	numerous	20.5	4.0	0
	2	numerous	22.7	2.7	0
	Av.	numerous	21.6	3.3	
3	1	—[b]	23.7	5.7	0
	2	—	30.2	3·0	0
	Av.	—	26.9	4.3	0
4	1	—	no increase in plaque count		0
	2	—	no increase in plaque count		0
	Av.	—	no increase in plaque count		0

[a] Average of four cultures in each virus dilution.
[b] Not observed.

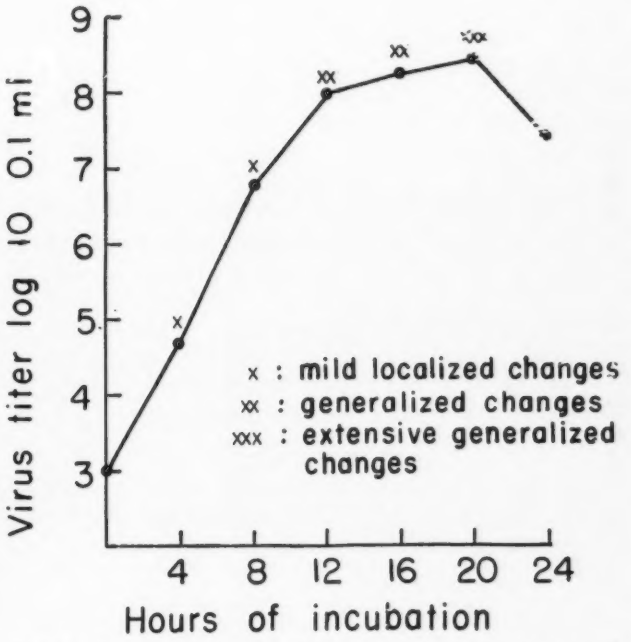

Fig. 3. Relationship of the intensity of cytopathogenic changes and magnitude of virus propagation.

control cultures inoculated with 10^{-5} virus dilution had an average of three plaques per culture.

Fig. 3 shows the relationship of the intensity of cytopathogenic changes to the magnitude of virus propagation. Significant increase of virus titer after four hours of incubation was accompanied by mild localized cytopathogenic changes. After 12–16 hours of incubation, the cytopathogenic changes were generalized and intensified. Virus titer increased to over five logs higher than the initial titer. Peak virus titer was reached after 20 hours of incubation, at which time the cytopathogenic changes were most extensive.

DISCUSSION

The seed virus retained a relatively high titer in chicken embryos and in cell culture system after ten passages in duck embryo fibroblast cultures. The cytopathogenicity and plaque-forming activity of the virus were neutralized by EE-immune serum but not by normal serum. These results indicate that the duck embryo fibroblast cell cultures were susceptible to the virus and that the system is suitable for virus propagation. The cell cultures may also serve for assay and investigation of the virus.

SUMMARY

A strain of eastern encephalitis virus originally isolated from duckling was carried ten passages in duck embryo fibroblast cultures. Cytopathogenic changes in inoculated cultures were observed in all ten passages. The tenth-passage virus formed plaques in the cell culture system. The cytopathogenicity and plaque-forming activity of the virus were neutralized by EE-immune serum but not by normal serum. Use of the cell culture system for virus assay was suggested.

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STUDIES ON THE ANTIGENICITY IN TURKEYS OF AN ERYSIPELAS BACTERIN* MADE FROM *ERYSIPELOTHRIX RHUSIOPATHIAE* STRAINS H_4 AND H_7

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ERYSIPELAS is an acute, contagious, septicemic disease mainly of turkeys and swine. It has also been reported in other domestic animals, in rodents, and in several species of fowl.

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* Dr. Salsbury's Erysipelas Bacterin is a trade mark of the product of Dr. Salsbury's Laboratories, Charles City, Iowa.

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Once the etiology was established, various vaccines and bacterins were reported and used for disease prevention. Among these, virulent cultures combined with anti-erysipelas serum, attenuated or avirulent cultures, and whole-broth inactivated cultures. Major attention has been given to the inactivated bacterins because of their safety and immunogenicity.

Traub[7] reported on an erysipelas bacterin of whole-broth culture, formalin-inactivated, aluminum-hydroxide-precipitated, and concentrated. This type of bacterin, prepared from a mixture of highly immunogenic strains, was used successfully all over the world. Reports on its efficacy in turkeys and swine were made by Dickinson *et al.*,[4] Cooper *et al.*,[2,3] and Phillips.[6] The *Erysipelothrix rhusiopathiae* strains most commonly used in this type of bacterin are the R variant strains Yena-36, SE-9, SE-25, RO-12, and AN-4, incorporated in equal proportions in the final product.

Nardelli,[5] and Berarducci[1] reported on an aluminum-hydroxide-precipitated bacterin containing equal proportions of two highly immunogenic *E. rhusiopathiae* S variant strains H_4 and H_7. Potency tests conducted by the authors gave excellent results with mice and swine. This bacterin was found to be slightly superior to the virulent culture plus serum and live attenuated vaccines.

Since there are no published data on the efficacy of this S variant bacterin in turkeys, the present paper reports on studies in field and laboratory comparing the antigenicity of this bacterin with that of a commercial mixture of five R variant strains.

MATERIALS AND METHODS

Strains characterization. The *E. rhusiopathiae* strains H_4 and H_7 belong to the serotype B. On tryptose agar they form small, smooth, transparent, convex, dew-drop-like colonies. Both strains grow vigorously in tryptose broth, with or without addition of serum, forming a slight sediment. Morphologically they appear as short, slender, nonmotile gram-positive rods. They produce acid without gas from lactose and dextrose, but do not ferment sucrose, maltose, mannite, or dulcitol. These strains are H_2S-positive, unagglutinable by trypaflavine, and,

in common with the R variants, hemagglutinate chicken erythrocytes. They kill mice within 96 to 144 hours, but are apathogenic to turkeys and swine.

Bacterin preparation. The bacterin was made essentially according to the method of Traub.[7] Broth cultures of the H_1 and H_7 strains, in equal proportions, were pooled, formalin-inactivated, aluminum-hydroxide-gel-precipitated, and concentrated. Prior to use, each lot of bacterin was tested for sterility, safety and potency in mice. This bacterin is referred to herein as bacterin A.

Commercial bacterin. A commercial aluminum-hydroxide-precipitated and concentrated erysipelas bacterin referred to herein as bacterin B, made from equal proportions of Yena-36, SE-9, SE-25, RO-12 and AN-4, was purchased and used in these trials. This bacterin was identical to that of A in its final bacterial concentration, adjuvent proportions, and methods of preparation. Both bacterins were equally effective when compared in mice.

Turkey safety and potency test of Erysipelas bacterins A and B and duration of immunity under laboratory conditions. The birds used were 180 Broad Breasted Bronze turkeys, nine weeks old, from an erysipelas-susceptible flock. The birds were divided into three equal groups, kept on the floor in separate pens, and fed a turkey growing ration containing Histostat-50, a blackhead preventive that does not interfere with bacterin antigenicity. One group was vaccinated with bacterin A, and one with commercial bacterin B. The third group served as unvaccinated susceptible controls. The usually recommended dose of 2.0 ml per bird was administered subcutaneously on the dorsal portion of the neck.

Beginning with the second postvaccination week and thereafter at various intervals, ten birds in each group were challenged. For the challenge at the second and third postvaccination weeks, each bird was given intravenously 0.5 ml of a lyophilized culture of the virulent *E. rhusiopathiae,* strain LN-261, diluted 1:10. From the sixth through the sixteenth postvaccination weeks, each bird was given intramuscularly, 1.0 ml of a suspension of infected turkey liver derived from birds

Table 1. Challenge results and duration of immunity in turkeys vaccinated with erysipelas bacterins A and B under laboratory conditions.

Challenge, week following vaccination	Erysipelas bacterins		Susceptible unvaccinated controls
	A	B	
2	0/10[a]	0/10	5/10
3	0/10	0/10	2/10
6	0/10	0/10	4/10
9	0/10	0/10	4/10
12	0/10	0/10	8/10
16	0/9	1/9	3/5

[a] No. dead/no. challenged.
Seven turkeys died during the 16-week period from nonspecific causes.

killed by the same culture. The birds were observed for seven days following challenge. The results are in Table 1.

Turkey potency test of Erysipelas bacterin A at one-half the recommended dose versus commercial B at regular dose. The birds, 30 Broad Breasted Bronze turkeys ten weeks old, were divided into three equal groups. One was vaccinated with bacterin A (1.0 ml per bird) and one with bacterin B (2.0 ml per bird). The third group served as unvaccinated susceptible controls. The bacterins were administered subcutaneously in the neck as described. As in the previous test, all three groups were challenged two weeks after vaccination with a 10% suspension of infected turkey liver. The results are in Table 2.

Turkey potency test of Erysipelas bacterin A under field conditions. The turkey field trials were conducted under our direct supervision. The flocks varied in size, breed, sex, and age. Flock susceptibility to erysipelas infection was determined

Table 2. Challenge results on turkey immunity two weeks after vaccination with erysipelas bacterins A and B under laboratory conditions.

Erysipelas bacteria	Dose ml/bird	No. dead/no. challenged	
		Vaccinated	Unvaccinated susceptible controls
A	1.0	0/10	10/10
B	2.0	1/10	

Table 3. Challenge results on turkey immunity two weeks after vaccination with erysipelas bacterin A under field conditions.

No. flocks vaccinated	Breed	Age (weeks)	Flock size	No. dead/no. challenged	
				Prevaccination	Postvaccination
1	Beltsville White	12	1500	4/10	0/10
2	Empire White	15	550	2/10	0/10
3	Broad Breasted Bronze	24	1100	5/10	0/10
4	Beltsville White	12	800	5/10	0/10

by removing 10 birds from each flock for prevaccination challenge. Susceptible flocks were then vaccinated. The bacterin was administered at the same site at 2.0 ml per bird, as previously reported. Two weeks after vaccination ten birds from each vaccinated flock were brought into the laboratory for challenge. The challenge was a 10% suspension of infected turkey liver, administered as previously reported. The birds were observed for seven days. The results are in Table 3.

RESULTS AND DISCUSSION

The results in Table 1 indicate that bacterins A and B were almost equally immunogenic for turkeys under laboratory conditions. The immunity in both groups was present two weeks after vaccination, and persisted for at least 16 weeks.

No untoward reaction to bacterin was observed in either group except for a mild local reaction that disappeared within a few weeks. None of the birds vaccinated with bacterin A showed signs of disease or died following challenge, while only one bird vaccinated with bacterin B showed signs and died. Upon challenge, all of the susceptible unvaccinated controls showed signs of disease and 26 birds died (47% mortality). The seven-day postchallenge period of observation was more than sufficient since mortality among the susceptible unvaccinated controls usually occurred within 24–96 hours. The difference in mortality between the two vaccinated groups may not be sufficient to justify the superior antigenicity of bacterin A to that of B.

The results in Table 2 indicate the antigenicity of bacterin A was still high since bacterin A at reduced dose induced better protection than bacterin B at the standard dose. Im-

munity was present in both groups two weeks after vaccination. There were no signs of disease or mortality among the birds vaccinated with bacterin A, whereas 10% of the birds vaccinated with bacterin. B showed signs and died. Mortality was 100% among the susceptible unvaccinated controls, indicating the severity of challenge.

Table 3 shows results under field conditions with bacterin A only. Four flocks (3,950 turkeys) were vaccinated. On challenge the birds were completely resistant two weeks following vaccination. None of the vaccinated challenged birds showed signs of disease or died. Mortality was 40%, and morbidity 100% among the susceptible unvaccinated controls. It is evident that erysipelas bacterin A elicited an excellent protection in turkey flocks under field conditions. Even though the susceptible unvaccinated controls were challenged two weeks prior to the vaccinated groups, we are certain that immunogenic changes could not have occurred in such a short time.

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SUMMARY

1) The characteristics of *Erysipelothrix rhusiopathiae* S variant strains H_4 and H_7 were described. From these was developed an effective bacterin, formalin-inactivated, aluminum-hydroxide-gel-precipitated, and concentrated. This bacterin (A) was compared with a commercial erysipelas bacterin (B), a mixture of five R variant strains.

2) Potency test data on A versus B in turkeys under laboratory conditions indicated a slight superiority of the former.

3) Turkeys immunized under laboratory conditions with A at one-half the recommended dose compared to B at the regular dose showed a greater resistance to challenge.

4) Data on the application of bacterin A in turkeys under field conditions indicated that the bacterin had induced a strong immunity in the vaccinated birds.

5) The erysipelas bacterin made only of strains H_4 and

H_7 appeared superior to the commercial bacterin containing five R variant strains.

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FURALTADONE WATER MEDICATION AGAINST NATURALLY INDUCED SALMONELLA PULLORUM INFECTION IN STRESSED FLOOR-PEN BROILERS[a]

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FURALTADONE water medication has been demonstrated to be effective against pullorum disease and fowl typhoid in chicks and poults, and paratyphoid infections in poults caused by *Salmonella typhimurium* and *S. heidelberg*.[1,2] All experimental work in connection with these observations was conducted in wire floor batteries under carefully controlled conditions. One view, however, is that the only really satisfactory test of a drug is the performance it gives under field

[a] South Carolina Agricultural Experiment Station, technical contribution No. 362. Published with permission of the director.

or floor-pen production conditions, where normal densities of bacterial and parasitic infections are encountered.[3] Thus, this experiment was undertaken to determine the anti-Salmonella properties of furaltadone water medication in chickens maintained in floor pens under conditions of stress.

MATERIALS AND METHODS

One hundred 2-hour-old broiler chicks in each of 4 floor pens were exposed to pullorum disease by introducing into each pen 30 marked chicks that had been given ½ ml of a broth culture of *S. pullorum* per os. The chicks were from a hatchery with no recent history of Salmonella infection. After 10 days, the so-called "seeder" chicks were removed.

It had been determined by preliminary trials that stressing the chicks by chilling was essential to cause mortality in the exposed groups. Brooders were adjusted to provide 80°F at the edge of the heated area. On the fifth day brooders were shut off for 6 hours. This reduced heat period was increased daily so that, on and after the 21st day, no brooder heat was supplied. On the 17th day, chicks of all groups were water-vaccinated against Newcastle disease and infectious bronchitis. Brooder temperatures were lowered rather than increased during the "take" period.

Causes of deaths of all birds were determined by routine diagnostic procedures. This included culturing heart sacs, livers, and spleens. Triple-sugar-iron (TSI) agar (Difco) slants proved a valuable differential medium. Suspicious TSI reactions were further processed biochemically for citrate and urea utilization, indole production, motility, and reactions on gelatin and sugars. A typing kit was useful in determining serological classification. A record of mortality was made daily, and at the termination of the experiment, feed consumption and body weights were summarized. All carcasses were classified as either fit or unfit for food. Inspection disposition in general considers extensive lesions in one or more internal organs and poor fleshing to be sufficient causes for condemnation.

Furaltadone medication of two pens of 100 chickens consisted of ½ g of pure drug (0.0132%) per gal. of drinking

Table 1. Summary of results: 8 weeks of age

Medication	Chicks per unit	Died (%)*	Av. body weight survivors (lb.)	Survivors inedible** (%)	Percent chicks edible of started
Furaltadone water medication, 0.5 g (0.0132%) per gal. to 14th day; 0.25 g (0.0066%) per gal. from 14th to 56th day	100 (Rep. 1)	6	2.58	1	93
	100 (Rep. 2)	11	2.66	1	88
Unmedicated infected	100 (Rep. 1)	55	1.89	55	20
	100 (Rep. 2)	46	1.69	74	14

* All deaths were attributed to pullorum disease except one unmedicated bird and two medicated birds affected with air sac lesions from which *E. coli* was isolated.

** Most of the condemnations were due to an extensive, purulent pericarditis.

water for the first 2 weeks. This was then reduced to ¼ g of drug (0.0066%) per gal. for the remainder of the 8-week experiment. Medication, in metal commercial waterers, was prepared and changed daily. Two additional infected pens were not medicated. No uninfected birds were maintained.

RESULTS AND DISCUSSION

Results are given in Table 1. The differences in mortality, body weights, and fitness for food between furaltadone-medicated and unmedicated groups were most dramatic. Medicated birds were relatively uniform in size. While large well developed birds were present in the unmedicated groups, the low average body weights were due to lack of uniformity and the presence of small birds in poor flesh.

SUMMARY AND CONCLUSIONS

The data of the experiment indicate that furaltadone water medication is an excellent drug for the treatment of pullorum disease in broilers maintained in floor pens under conditions of stress. This confirms similar findings conducted in battery pens under controlled conditions.

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FURTHER OBSERVATIONS CONCERNING ROUS VIRUS NEUTRALIZING ANTIBODIES AND NEOPLASIA. II. IN BREEDER AND EXPERIMENTAL FLOCKS

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LITERATURE on neoplasia and Rous virus neutralizing antibodies (RVNA) in avian populations was reviewed in a previous paper.[8] Other information presented indicated that farm flocks experiencing significant losses associated with visceral lymphomatosis also appeared quite susceptible to the Rous virus or to an antigenically related agent on the basis of the presence of RVNA.

Reported herein are further studies of this problem in breeder and experimental flocks.

As in the previous paper,[8] the susceptibility of a population to the Rous agent or an antigenically related agent was assessed on the presence of RVNA in the sera. Susceptibility to visceral lymphomatosis and other tumors was based on the per cent of chickens lost due to tumors determined through necropsy. Available tumor histories of the parent generations are summarized in the appropriate tables.

METHODS

The presence of RVNA was determined by a modified

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subcutaneous method described previously.[8] Sera were held in a hard freeze at –25°C or in dry-ice chest at –70°C until serum neutralization tests could be completed. The amount of Rous virus employed in the neutralization tests varied from about 5 to 35 MID_{50} (based on the subcutaneous inoculation method[8]).

All chickens that died were examined routinely for disease, and particularly for evidence of tumors. Observation periods for the different populations varied from 225 to 925 days.

A. Breeder Populations

Blood specimens for the RVNA test were collected from 5–10% of various White Leghorn pure-line and cross-line populations 8–12 weeks old. In some cases, adult populations were bled.

Beginning at one day of age and continuing for about 5 months, most of the populations tested were deliberately exposed to lymphomatosis-susceptible adult hens by rearing in adjacent pens. Hatches from 1954, 1955, 1956, and 1957 were included in this study.

Populations held until laying age in semi-isolation without deliberate exposure to adult hens were considered as not exposed, though some exposure may occur under such conditions. In some cases, the tumor susceptibility of populations reared in semi-isolation was compared with that of similar genetic populations that had not been deliberately exposed during the rearing period.

B. Experimental Populations

The inbred lines of White Leghorns used were maintained by personnel of the U. S. Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Michigan. Populations hatched in 1953 and 1954 were naturally exposed through rearing in the same building with adult populations and the employment of common caretakers. In addition, several lines of the 1953 hatch were inoculated at one day of age with lymphomatosis tumor materials. The inoculated populations were brooded and reared in separate quarters but unquarantined from the adult stock. All sera and the

corresponding tumor performance data were supplied by Dr. B. R. Burmester, of the Regional Poultry Research Laboratory.

RESULTS

A. Breeder Populations

All 1954–55 hatches (Table 1) receiving a planned exposure, except D and B, experienced at least a 5% tumor loss. Each of these deliberately exposed populations except C developed a 10% level or higher of RVNA. Genetically similar populations FE and FI (Table 1) were found to have 44 and 72% of the respective sera positive for RVNA. Tumor losses were 17.7% in the exposed FE chickens, and only 1.6% in the isolated FI populations. RVNA were demonstrated in 87% of hatch GE (Table 1), whereas actual tumor loss was 21%. Population GEA, with the same genetic pattern as GE and located at a separate poultry ranch, experienced 8% mortality due to tumors and a 37% occurrence of RVNA.

The data for the 1956 hatch are also summarized in Table 1. Only two of these populations (6E and 11E) suffered tumor losses of over 5%; only 3 groups (3E, 4E, 10E) had over 10% of the flock with sera positive for RVNA. The FE and GE populations, with previous histories of tumor susceptibility, developed no RVNA, and only 3.5 and 1.0% tumor losses, respectively.

In the 1957 hatch (Table 1), only 3 populations (1I, 3I, 3E) had over 10% of the respective flocks positive for RVNA, whereas the respective tumor losses were 0.2, 7.4, and 26.4%. Population 6E lost 6.8% of the flock with tumors, and 5.8% had sera with RVNA. Tumor losses were greatest in the populations with a history of tumor susceptibility (3I, 3E, 6E). Two genetically similar populations, 3E (deliberately exposed) and 3I (not deliberately exposed), may have reflected the relative effect of exposure severity, with respective losses of 26.4 and 7.4%.

B. Experimental Flocks (Table 2)

Thirty to 89% of the sera from chickens in naturally exposed lines 6, 9, and 15, from the 1953 and 1954 hatches, were positive for RVNA. Line 7 had 7–9% sera positive for RVNA.

Table 1. The prevalence of tumors[a] and Rous virus neutralizing antibodies (RVNA) in breeder populations.

Population	Bleeding age (days)	Period observed (days)	Total No.	RVNA Results No. (+)	RVNA Results % (+)	Losses due to tumors No.	VL[b]	NL[c]	other[d]	%	Remarks[e]
1954-55 hatches											
A	94	515	1351	20/70[f]	28.5	102	85		17	7.5	DEX
B	94	515	270	8/50	16.0	9	9		0	3.3	DEX
C	94	515	58	1/30	3.3	6	5		1	10.3	DEV; TSH[g]
D	94	513	70	8/32	25.0	0	0		0	0.0	DEX
E	94	515	70	3/30	10.0	6	6		0	8.6	DEX
FE	305	225	62	11/25	44.0	11	11		0	17.7	DEX; TSH
FI	264	225	122	29/40	72.5	2	2		0	1.6	NDEX; TSH; SL:FE
GE	234	270	114	26/30	87.0	24	24		0	21.6	DEX;TSH
GEA[h]	210	240	350	7/19	37.0	28	28		0	8.0	DEX; TSH; SL:GE
1956 Hatch											
1	68	828	767	0/44	0.0	16	8	5	3	2.1	NDEX
2	68	828	691	0/46	0.0	13	10	2	1	1.9	NDEX
3E	85	843	535	7/54	13.0	2	2	0	0	0.37	DEX
4E	85	300	577	6/50	12.0	7	5	2	0	1.2	DEX
5E	85	300	93	0/21	0.0	1	1	0	0	1.1	DEX
6E	85	300	106	2/31	6.5	1	0	1	0	9.4	DEX
7E	85	300	103	1/29	3.5	2	0	2	0	1.9	DEX
8E	85	300	92	1/25	4.0	1	0	1	0	1.1	DEX
9E	85	300	99	1/27	3.7	2	0	2	0	2.0	DEX
10E	85	300	102	3/26	11.5	1	1	0	0	1.0	DEX
11E	85	300	57	0/21	0.0	4	1	3	0	7.0	DEX
12E	85	300	101	1/23	4.3	1	1	0	0	1.0	DEX
FE	85	300	57	0/22	0.0	2	2	0	0	3.5	DEX; TSH
GE	85	300	99	0/24	0.0	1	1	0	0	1.0	DEX; TSH

(Continued)

Table 1 (Continued)

1957 hatch											
1I	84	370	2133	16/60[b]	26.6	5	2	2	1	0.2	NDEX;TRH
2I	84	370	1350	1/44	2.3	6	4	1	1	0.4	NDEX;TRH
3I	84	370	108	6/20	30.0	8	6	1	1	7.4	NDEX;TSH
3E	63	370	34	1/8	12.5	9	6	2[i]	1	26.4	DEX;TSH
4E	63	925	946	1/48	2.1	11	5	1[i]	5[j]	1.2	DEX;TRH
5E	63	925	620	4/46	8.7	12	5	0	7[k]	1.9	DEX; TRH
6E	63	370	74	1/17	5.9	5	1	3	1	6.8	DEX;TSH

[a] Includes all types of tumors observed. No histopathologic studies made.
[b] Visceral lymphomatosis.
[c] Neural lymphomatosis.
[d] Includes all tumors except visceral lymphoid neoplasms.
[e] DEX, deliberately exposed; NDEX, not deliberately exposed; TSH, tumor-susceptible history; TRH, tumor-resistant history; SL, same line as.
[f] Numerator refers to number positive, and the denominator to the number of tests.
[g] Tumor losses of more than 10% in previous generations.
[h] Hatched and exposed at a different station than flock GE.
[i] One neural case also had VL.
[j] Tumor sites: 1 in kidney; 1 in ovary; 1 in proventriculus and intestine; 1 in ovary intestine and mesentery; 1 in ovary, oviduct, intestine and mesentery.
[k] Tumor sites: 3 in ovary; 2 in skeletal muscle and skin; 1 in ovary and mesosalpinx; 1 in kidney and oviduct.

In 1953, lines 9 and 15 respectively experienced 13.8 and 31.3% visceral lymphomatosis (VL) losses. In 1954 the respective tumor losses were 13.7 and 4.8%. Tumor losses for lines 6 and 7, hatched in 1953 and 1954, ranged from 3.3 to 3.8%. When the 1953 hatch was inoculated with lymphomatosis virus, 82.6% of the sera from line 6 were positive for RVNA, whereas 13.7, 12, and 11.5% of the sera from lines 7, 9, and 15, respectively, were found positive for RVNA.

All these lines lost 20% or more of the flock by VL when inoculated with VL virus materials except line 7, which lost only 2.9%.

DISCUSSION

Earlier data[8,11,12] collected from field flocks with significant tumor losses suggested a direct correlation between the occurrence of RVNA and tumor losses other than those associated with neural lymphomatosis. It is interesting to note (Table 1) that two genetically similar populations, FI and FE, one isolated from and the other deliberately exposed to tumor-bearing hens, both had such a high occurrence of RVNA and yet experienced such different tumor losses. Based on the presence of RVNA the FI isolated chickens must have experienced infection with a Rous agent or one antigenically related, possibly by transmission through the egg or by inapparent contact. Burmester *et al.*[4] reported that the Rous agent can be transmitted by contact, resulting in the development of Rous tumors; however, post-mortem reports on FI and FE poulation did not refer to significant numbers of Rous-like tumors. It is possible that the Rous or an antigenically related agent might stimulate the formation of RVNA without the apparent development of tumors. Carr[5] reported that the Rous agent may remain latent a year before tumors develop. Kenzy[10] presented data suggesting that an agent antigenically related to Rous, or the Rous agent, appears to be transmitted through the egg and thus serves as a source of exposure to contact chicks. Since, however, Carr[5] has shown that RVNA, which may be passed through the egg yolk to the chick, persists for only a few weeks, we are most likely not dealing with such antibodies. In an attempt to explain

Table 2. Presence of visceral lymphomatosis and Rous virus neutralizing antibodies in adult populations at Regional Poultry Research Laboratory[a]

	Naturally exposed populations								Inoculated populations			
	1953 hatch				1954 hatch				1953 hatch			
	Prevalence of RVNA[b] in sera		Prevalence of VL[c]		Prevalence of RVNA in sera		Prevalence of VL		Prevalence of RVNA in sera		Prevalence of VL	
Line	No. (+)	% (+)	No. (+)	% (+)	No. (+)	% (+)	No. (+)	% (+)	No. (+)	% (+)	No. (+)	% (+)
6	51/57[d]	89.5	3/82	3.7	28/51	54.9	3/89	3.4	38/46	82.6	32/91	35.2
7	4/44	9.1	3/89	3.4	4/57	7.0	4/105	3.8	7/51	13.7	3/101	3.0
9	27/50	54.0	12/87	13.8	26.45	57.8	13/95	13.7	6/50	12.0	23/113	20.4
15	18/60	30.0	31/99	31.3	21/53	39.6	4/84	4.8	6/52	11.5	32/108	29.6

[a] Serum specimens and tumor performance data made available by Dr. B. R. Burmester, Regional Poultry Research Laboratory, East Lansing, Michigan.
[b] Rous virus neutralizing antibodies.
[c] Visceral lymphomatosis (neural type excluded).
[d] Ratio of number of positive to total tests.

the apparent direct correlation between the presence of RVNA and visceral lymphomatosis in certain populations, Duran-Reynals *et al.*[6] and Kenzy[11] suggested that the lymphoid and Rous agents may be antigenically related. Burmester[2] as well as Beard[1] reported information suggesting that there are antigenic components common to both the Rous and lymphomatosis agents.

It is also possible that the Rous and lymphoid agents are not related antigenically. Certainly the high occurrence of RVNA (73%) in an isolated population FI (Table 1) with a very low level of VL (1.6%) supports this concept, particularly when the same genetic strain FE (Table 1), which was exposed, developed a high level of RVNA (44%) and definite losses (17.7%) from VL. Likewise, the VL losses of 12% in a tumor-suseptible line (RPL 15) without the presence of demonstrable RVNA might be explained in the same manner.[12]

Rubin's observation[13] of a resistance-inducing factor for the development of Rous virus plaques in a tissue-culture system associated with VL virus may be significant in interpreting these data. Possibly, this interference may influence the progress of infection in the host, depending on the timing of exposure to these agents, with an effect on tumor response and the development of neutralizing antibodies.

The apparent lack of correlation between RVNA and tumor losses in certain populations (B, D, Table 1) may have been due to the early bleeding age, since Duran-Reynals described the development of RVNA in populations as age increases.[6] Inadequate sampling and limited numbers may also be of definite significance in some of the populations tested (Table 1).

The data (Table 2) suggest that certain avian populations, such as line 7, are quite resistant to the lymphoid agent(s) on the basis of a low level tumor formation, and apparently are likewise resistant to a Rous-like agent based on the low level of RVNA observed. The history of line 7 indicates a high relative resistance to VL and Rous sarcoma.[2]

We also noted in this study that a population apparently may be quite susceptible to a Rous-like agent, as suggested by

the high prevalence of RVNA, whereas, the low-level occurrence of lymphoid tumors would suggest a lack of susceptibility or inadequate exposure to the lymphoid agent. However, the low level of tumors might be expected in certain cases since Burmester[3] reported that clinically normal hens shed VL virus in the saliva and feces. The age of exposure[7] and amount of antibody[2] transferred in the yolk are also considered significant. Certain of our difficulties in the interpretation of data arise because we do not thoroughly understand the factors related to the triggering and formation of tumors.

SUMMARY

1. Various populations for breeder and experimental flocks studied 2–4 years exhibited a marked variation in the occurrence of tumors and prevalence of Rous virus neutralizing antibodies (RVNA).
2. There appeared to be a high-level occurrence of RVNA and tumors in certain populations, but not in others.
3. Usually, a low level of tumors appeared to be related to a low level of RVNA, though this is not always a consistent pattern.
4. The high prevalence of RVNA may be related to the widespread presence of the lymphoid agent(s) and the occcurrence of antigenic components common to the agents of Rous sarcoma and lymphomatosis. However, some of the data suggest that the Rous and lymphoid agents may not be antigenically related.
5. It is evident that controlled studies of the conditions associated with the development of RVNA are required to clarify their relationship to lymphomatosis.

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PROGRAM OF THE 10th ANNUAL WESTERN POULTRY DISEASE CONFERENCE FEBRUARY 1, 1961, DAVIS, CALIFORNIA

Some papers presented at the conference are printed elsewhere in AVIAN DISEASES or in brief form below.

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W. W. Sadler
Department of Public Health, School of Veterinary Medicine, University of California
Davis, California

DIAGNOSIS OF ORNITHOSIS
E. M. Dickinson
Department of Veterinary Science, Oregon State University
Corvallis, Oregon

INFECTIOUS SYNOVITIS
R. R. Chalquest
Department of Veterinary Science, Washington State University
Pullman, Washington
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CANDIDIASIS
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ESCHERICHIA COLI INFECTIONS
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FACTORS AFFECTING *HEMOPHILUS GALLINARUM* INFECTION
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Martin Goetz
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USE OF INFECTIOUS SINUSITIS AGGLUTINATION TEST
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AVIAN ENCEPHALOMYELITIS PROBLEMS AND CONTROL
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DeKalb Agricultural Association
DeKalb, Illinois

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Kimber Farms, Inc.
Niles, California
Avian Hysteria

ADEQUACY OF CRITERIA USED TO EVALUATE THE RESULTS OF POULTRY DISEASE RESEARCH

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The advent of compulsory poultry meat inspection not only has had a tremendous impact on poultry producers and poultry processors, but also has placed added responsibility on the shoulders of research poultry pathologists. Prior to January, 1959, a bird was salable if it was alive and in reasonably adequate flesh when it was to be marketed. Now, however, a bird must also be classed as wholesome by the Inspector at the time it is slaughtered. The very definition of the word "wholesome" is the subject of controversy since it is influenced not only by the presence or absence of disease-producing agents but even more by aesthetics.

In red-meat inspection, working definitions of what is wholesome and what is not wholesome have evolved over the past 50 years; they are no longer a subject of controversy among the groups affected. The Agricultural Marketing Service has a legal as well as a moral responsibility to enforce the law as it is written and to condemn carcasses and tissues that do not come up to the standards of wholesomeness that are presently accepted by the general public.

The classical criteria of the efficacy of vaccination procedures have been: 1) antibody response; 2) degree of symptoms shown following challenge; 3) survival following challenge; and 4) in some instances gross pathology at time of sacrifice (usually an arbitrarily selected time following challenge). The classical criteria of the efficacy of therapy regimes have been: 1) reduction of severity of symptoms; 2) clinical recovery; 3) survival; 4) in some few instances weight gain and feed conversion; and 5) in rare instances the degree of resolution of gross lesions. Rarely considered is the relation of time of vaccination and subsequent challenge as well as time of therapy to the "economically critical period" of meat birds. For instance, in chicken fryers to be slaughtered at two months of age, duration of high antibody levels is of little moment whereas the timing of the vaccination as well as the choice between live versus killed vaccine may be all-important. Although certain types of live vaccine may give high antibody titers and result in lasting immunity the introduction of this attenuated virus may result in its acting synergistically with another agent that may be present in the tissues of the bird to cause lesions that result in the carcass being unwholesome at the normal market age although the lesions might resolve if the bird were held longer. A hypothetical example might be a flock of birds harboring Mycoplasma without lesions or disease but vaccinated with infectious bronchitis virus, resulting in the development of a pathology not resolved before slaughter. As to therapy, one might hypothesize that there are drugs or antibiotics that may not do a very good job of suppressing symptoms (one of the main criteria for judging the adequacy of such agents) but may suppress the development of pathology sufficiently to prevent condemnation of the bird. Thus, our present concepts of types of vaccines used, timing of the vaccination, and regimes of therapy

Table 1. Comparison of symptoms of disease in live birds to degree of gross pathology in the processed carcasses.

Severity of symptom	Ante-mortem		Post-mortem: Sufficient pathology to cause condemnation for unwholesomeness	
	Number	% of total	Number	% of live category
Adult chickens				
Extensive	215	10.28	187	86.98
Mild	689	32.93	497	72.13
None	1188	56.79	467	39.31
Totals	2092		1151	53.31
Chicken fryers (market age)				
Extensive	59	4.31	58	98.30
Mild	464	33.94	412	88.79
None	844	61.74	677	80.21
Totals	1367		1147	83.91

should be re-evaluated in light of this now important standard of "wholesome at the economically critical period."

Table 1 summarizes a comparison between the ante-mortem decision as to the degree of symptoms observed in live birds and the post-mortem decisions as to the health and wholesomeness of the carcasses as evidenced by the degree of pathology observed in 2,092 adult chickens and 1,367 chicken fryers. These were randomly chosen individuals from 24 laying flocks and 20 fryer flocks that were experiencing disease outbreaks and were under various regimes of therapy.

Of 1,188 adult chickens free of any detectable symptom before slaughter, 467 (39%) were found on post-mortem examination to contain lesions or pathology extensive enough to render them unwholesome and unfit for human food. Of 844 fryers passed at ante-mortem with no evidence of a symptom that would indicate disease or pathology, 677 (80%) were found on post-mortem examination to have sufficient pathology to render them unwholesome and unfit for human food. Similarly, of the 689 adult chickens and 464 fryers showing only mild symptoms, respectively 72 and 89% evidenced sufficient gross lesions to render them unwholesome. The vaccination program or therapy regime would be called a success on the basis that 1188 (57%) of the adult chickens and 844 (62%) of the chicken fryers showed no visible symptoms of disease at examination, or on the basis that 1877 (90%) of the adults and 1308 (96%) of the fryers showed none or only mild symptoms. Nevertheless, such an evaluation would have been completely misleading as to the effect on the marketability of these meat birds—the primary objective of raising them—since 53% of the adults and 84% of the fryers evidenced sufficient pathology to render them unwholesome and cause their condemnation.

A greater emphasis must be placed on the "economically critical" period of a given bird's life. With the breeder or layer this would of course be the period of egg production when this bird must be: 1) alive; 2) in reasonable health; and 3) producing eggs of the quantity and type desired. With meat birds the critical period would be the age at which

they are normally marketed: chicken fryers, 8-10 weeks old; turkey fryers, 13–16 weeks; young turkey hens, 19–22 weeks; young turkey toms, 24–27 weeks.

All husbandry practices and vaccination and therapy regimes must be aimed at a climax in these periods, when these individual birds must be: 1) alive; 2) in reasonably good flesh; and 3) free of pathology that would render the carcass unwholesome. It is this last requirement that must receive increasing attention from the poultry pathologist. It is, therefore, readily evident that a separate approach must be made in the research aimed at layers and breeders versus that aimed at meat birds. It behooves the individual research pathologist to recognize the import of this now all-important criterion of "wholesomeness" upon his past and present research and upon the advice he gives to the meat-bird producer.

SANITATION AS AN APPROACH TO CONTROL OF PARATYPHOID AND PARACOLON INFECTIONS

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Good flock and hatchery sanitation programs have gone far toward preventing the spread of paratyphoid and paracolon infections from poultry breeding flocks to the progeny. These measures have been particularly helpful for the control of these diseases in turkeys.

Emphasis is being placed on effective rodent and reptile control, the production of clean hatching eggs from clean nests, formaldehyde-gas fumigation of turkey eggs on the farm right after they are gathered, the use of new or fumigated egg cases, and repeated fumigation of eggs at the hatchery. It has now become common practice to use heavier fumigation than formerly and to fumigate the eggs early to help prevent shell penetration by bacteria from fecal contaminations.

A leaflet ("Fumigation of Hatching Eggs," Dr. D. E. Stover, Poultry Disease Specialist, reprinted from Calif. Dept. of Agriculture Bulletin, *46*, 30-33) on the fumigation of hatching eggs is available to those desiring more detailed information.

COMMENTS ON DIAGNOSIS OF ORNITHOSIS

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The need for poultry-disease diagnosticians to give greater attention to the diagnosis of ornithosis in birds was stressed. Tissues and lesions from birds suspected of containing ornithosis virus should be properly prepared and inoculated into mice or chicken embryos. If the characteristic reaction is not noticed in the test animal on the first passage, at least three blind passages without recovery of the virus should be made before a negative diagnosis is rendered. Macchiavello and Giemsa stains of microslide impressions from the surface of organs or lesions infected with ornithosis have proved to be effective tools for observing the characteristic Levinthal-Cole-Lillie (L-C-L) bodies associated with this infection.

PRELIMINARY OBSERVATIONS ON AVIAN HYSTERIA

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During the last two years a flock condition of hyperexcitability has been observed in laying hens and replacement pullets. It has been called "avian hysteria" to differentiate it from flightiness normally observed in White Leghorns. The first cases were observed in the summer of 1959. The cases reported increased in frequency in the late spring and summer of 1960.

Historically, cases were reported as having occurred in 1958; and from descriptions, current cases of hysteria may be identical with a condition referred to as "snake in the house" and thought to be caused by snakes or predatory animals invading the house.

Hysteria is usually of sudden onset, although a period of increasing nervousness has been reported. Affected chickens utter frightened cries and run at random, seeking shelter. In large pens this results in milling. Individual chickens seldom move very far, so that a wave of excitation moves down the house. Before subsiding, the wave of excitation often runs the length of the building and returns to reafflict the original birds, which in the meantime have become quiet.

In hysteria the excitation travels slowly from bird to bird compared with simultaneous "takeoff" of a pen of flighty pullets. The presence of a man in the pen has no effect on the milling. Each episode of excitation usually begins spontaneously within the flock; however, loud noise or rapid motion may initiate it. The period between episodes may vary from 2 minutes to many hours; the duration of each may be from 10 to 40 seconds.

Mortality is primarily the result of piling, and occasionally from tears inflicted by claws. Even though injuries are severe, cannibalism is not a problem.

Feather eating and lack of feathers appear to accompany hysteria, and feathers regrow on recovery. Recovery occurs in most cases within 6 weeks, and the diversity of the treatments that are followed by recovery complicates the search for a cause. Prolonged symptoms, lasting six months, have occurred in at least one case.

Hysteria has been observed in all the major strains of Leghorns and in meat-strain replacement pullets. The ages at onset in 10 cases were: 14–18 weeks in 6 cases and 8½–12 months in the other 4. In 19 additional cases, age at onset was 12–18 weeks in 15 cases and 7½ months in the other 4. These cases plus 3 others (age not recorded) occurred on floor with litter. Whether the same thing occurs on wire is not definitely known, because the milling and hiding cannot be observed. There are reports of cases in which hysterical pullets did not "quiet down" when placed in cages. In hens a severe drop in production is observed, apparently because the hens are hiding and not eating.

There is evidence that the condition is not contagious. In as many as half the cases reported in pullets, other chickens of the same age, older birds, and in some cases younger birds were housed on the same premises, often in adjacent pens, and failed to show evidence of hysteria prior or subsequent to the onset in the reported case. In two of the adult cases reported, the affected hens were placed in pens with normal hens without spread of hysteria to the normal hens. There are no gross lesions of

infection on post-mortem. Histological examination of brain from one case (Dr. Sanger—Wooster, Ohio) failed to show lesions. Differences observed in split broods, and its concurrent appearance in many strains, reduce the likelihood of hereditary cause and suggest some sort of threshold mechanism as the primary cause.

No specific treatment is known. Serpasil at 2 lb/ton (levels of drug unknown) has not been effective in alleviating symptoms. This condition has been so spectacular that it has been impossible to secure controlled treatment trials. Empirically, distracting palatable feeds such as greens or good-quality alfalfa hay combined with added B vitamins, and good natural vitamin sources such as brewer's yeast, have been given. Debeaking or detoeing growing pullets has occasionally appeared to help. Keeping the noise level up, particularly with normal male chicken sounds, has been tried with reported success. Breaking groups of laying hens into smaller lots and mixing with unaffected birds gave satisfactory results in a few cases. Treatment with the vitamins, yeast, and distracting feeds has been followed by recovery. A few cases in which no treatment has been given also recovered, however. At least one case continued for six months (until the onset of cold weather) before recovery.

Neither the cause of hysteria nor any means of prevention is known. Reports of cases have come from all parts of the United States. The total number of cases has been small, but most are spectacular and highly disturbing to the poultryman.

LIMITED STUDY WITH HAEMOPHILUS CULTURES

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Received 10 Feb. 1961

The use of *Haemophilus gallinarum* bacterins is gaining momentum in Southern California. Some poultrymen claim beneficial results following their use, and others claim no benefits whatsoever.

To test these reports *Haemophilus* bacterins trials were conducted on our research farm.

Material for challenge and for the bacterins or vaccines was produced by growing *Haemophilus gallinarum* previously isolated from a diagnostic accession, in the allantoic cavity of ten-day-old embryos. Allantoic fluid and the membranes for inactivated vaccine were harvested aseptically after two days of incubation and ground for immediate use. The organisms were treated with 0.25% formalin, and held 24 hours at 35°F, and formalin was added to 0.5% final concentration. The number of organisms in the suspensions was not determined.

The experiments and results are outlined in Table 1.

In Experiment I, 22 12-week-old White Leghorn pullets were inoculated intramuscularly (IM) with 1 ml of a live virulent *Haemophilus gallinarum* embryo culture. Thirty similar birds served as uninoculated controls.

By the fifth day post-inoculation, all principals were showing typical symptoms of coryza, with swollen sinuses, swelling and edema of the face, nasal discharge, and watery eyes. Two days later, two of these

Table 1. Design and results of experiments with *H. gallinarum* cultures or bacterins.[a]

Experiment no.	Amount	No. of birds	Birds with coryza	4-wk inoculation material	Challenge with virulent *Haemophilus gallinarum*: Time since first inoculation (wk)	Challenge with virulent *Haemophilus gallinarum*: Results (a)
#1						
Live culture	1 ml IM	22	22	LC (2 drops[b])	7	0/20
						24/29
	0	30	0			
#2						
Inactivated Bacterin	1 ml IM	81	0	0	3	most/111
						35/45
	0	30	0	0	4	23/28
#3						
Live culture	1 ml IM	20	0	LC (2 drops[b])	4	0/20
		20	0	0	8	0/20
Inactivated Bacterin	1 ml IM	20	0	Bact	4	0/20
		20	0	0	8	0/20
Controls		4	0	0	4	2/4
		5	0	0	8	4/5

[b] These vaccinates were inoculated with one drop in nostril and one drop in eye as indicated. Controls were not inoculated.

[a] 0 = no. inoculation or signs. LC = live *H. gallinarum* culture. Bact = formalinized bacterin.

(a) Fraction = birds with coryza/total.

birds were necropsied and cultured. *Haemophilus gallinarum* was isolated from the turbinates and sinus of each; both were culturally PPLO-negative and had negative PPLO agglutination. All the test birds were medicated by intramuscular injection of 1 ml Sulfamethazine, and one week later 1 ml of Terramycin in oil was inoculated subcutaneously. Within four weeks all the test birds were negative symptomatically and were challenged with one drop in the eye and one drop in the nostril of a live virulent *Haemophilus* culture. No symptoms of coryza or other illness were observed during the next three weeks, after which time the remaining test birds and the uninoculated controls were challenged with a virulent live culture of *H. gallinarum*, one drop each in the eye and in the nostril. One week later, 24 of the controls were showing symptoms infectious coryza, 5 were negative, and one was dead (picked). No symptoms were observed in the previously inoculated (test) group. Necropsied specimens were PPLO-negative by culture, but *H. gallinarum* was recovered from the sinuses and turbinates.

Experiment 2 consisted of 18-week-old White Leghorn cockerels, 81 vaccinates and 30 uninoculated controls. The vaccinates received 1 ml IM of formalin-inactivated *H. gallinarum* bacterin.

The two groups of birds were kept in separate quarters for three weeks, during which period no signs of illness were observed in either group. Then each bird was challenged with a virulent culture of *H. gallinarum*, one drop each into one eye and one nostril.

After four days, typical symptoms of infectious coryza were observed in most of the birds in each flock, and on the 7th day post-challenge each bird was individually examined for signs. Of 45 vaccinates, 35 showed signs of coryza; of 28 controls, 23 had typical signs.

A total of 8 birds from both groups had died from fighting during the 3-week holding period. Ten percent from each group were necropsied and cultured; *H. gallinarum* was isolated and PPLO was not.

Experiment 3 consisted of four groups of 20 White Leghorn 18-week-old pullets each.

The birds in two groups were injected intramuscularly with 1 ml each of a live virulent *Haemophilus* culture; birds in the other two groups were injected IM with 1 ml each of formalin-inactivated *H. gallinarum* bacterin.

Two weeks later one pen inoculated with live culture and one pen that had received bacterin were inoculated with a second dose of the respective inoculum.

Four weeks later all birds in each of the four groups, plus four uninoculated controls, were challenged with one drop of a live virulent culture of *H. gallinarum* in the eye and nostril. No symptoms of coryza were observed in any of the vaccinated birds, but two controls were positive, showing runny and crusted nostrils.

One month later, all the birds, plus five additional uninoculated controls, were again challenged with a live culture of *H. gallinarum*, one drop each in the eye and nostril. No symptoms were observed in any of the vaccinates. Four of the controls were positive, with runny, crusted nostrils. Cultures revealed *Haemophilus*, but no PPLO.

SUMMARY

Three experiments to test the efficacy of *Haemophilus gallinarum* vaccines or bacterins are reported. The use of living cultures gave satisfactory results while in the one of two trials the vaccinated birds were protected against intranasal challenge for a period of approximately four weeks.

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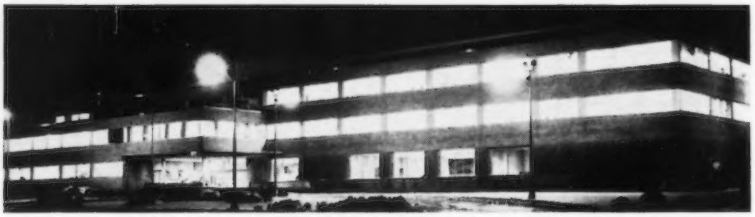

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In a study utilizing 640 Columbia Cross Chicks, Shumard[2] reports: ". . . FURACIN was effective in preventing mortality due to initial infections with *E. necatrix* if administered previous to and during the liberation of first generation merozoites."

"The drug did not interfere with the development of immunity to the coccidian nor did it produce any loss of feed conversion efficiency in the uninfected birds."

Reporting on the effectiveness of FURACIN in drinking water against *Eimeria tenella* infections in New Hampshires and White Plymouth Rocks, Johnson[1] concludes: "0.008% FURACIN is recommended since it affords maximum protection without interfering with the establishment of immunity or rate of growth."

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and for chronic respiratory disease (C. R. D.) of poultry . . .

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SUPPLIED: Carton of 100 Gm.

1. Johnson, C.: Paper presented at the 45th Annual Meeting of the Poultry Science Association, North Carolina State College, Raleigh, North Carolina, Aug. 7-10, 1956.

2. Shumard, R. F.: Paper presented at the First National Symposium on Nitrofurans in Agriculture, Michigan State University, East Lansing, Michigan, Sept. 28-29, 1956.

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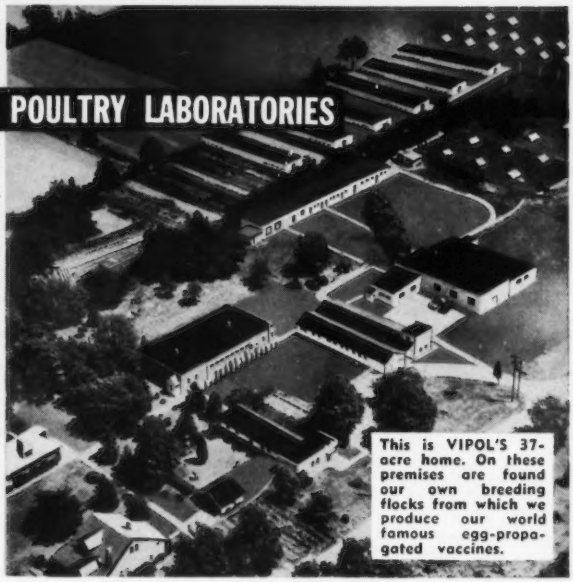

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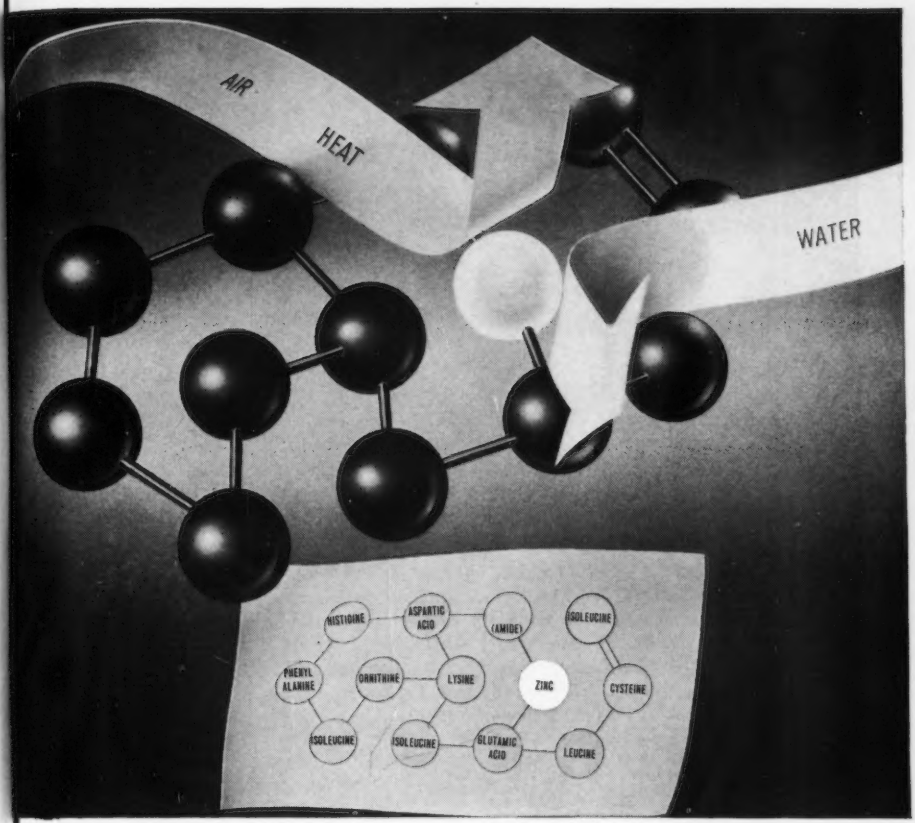

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References: 1. Plummer, A., Gaunt, R., and Belloff, G.: CIBA Research Laboratories. 2. Couch, J. R.: First Conference on the Use of the Tranquilizing and Antihypertensive Agent SERPASIL in Animal and Poultry Production, College of Agriculture, Rutgers, The State University, New Brunswick, N. J., May 7, 1959. 3. Weiss, H. S.: Ibid. 4. Burger, R. E.: Ibid. 5. Gilbreath, J. C.: Ibid. 6. Parker, E. L.: Second Conference on the Use of Reserpine in Poultry Production, The Institute of Agriculture, University of Minnesota, St. Paul, Minn., May 6, 1960. 7. Ringer, R. K.: Ibid. 8. Waibel, P. E.: Ibid. 9. Morrison, W. D.: Ibid. 10. Carlson, C. W.: First Conference, etc.

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